PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/775, A61K 38/16, C07K 16/18, G01N 33/68

A1

(11) International Publication Number: **WO 97/17371**

(43) International Publication Date: 15 May 1997 (15.05.97)

(21) International Application Number: PCT/US96/18136

(22) International Filing Date: 8 November 1996 (08.11.96)

(30) Priority Data:

60/006,395	9 November 1995 (09.11.95)	US
08/691,795	2 August 1996 (02.08.96)	US

(71) Applicant: ARCH DEVELOPMENT CORPORATION [US/US]; 1101 East 58th Street, Chicago, IL 60637 (US).

(72) Inventors: SCANU, Angelo, M.; 5825 South Dorchester, Chicago, IL 60637 (US). EDELSTEIN, Celina; 4800 South Chicago Beach Drive, Chicago, IL 60615 (US).

(74) Agent: HIGHLANDER, Steven, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

(81) Designated States: AL, AM, AT, AU, BA, BB, BG, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ISOLATION OF apo(a), COMPOSITIONS, AND METHODS OF USE

(57) Abstract

Disclosed are novel compositions comprising purification of active apolipoprotein (a), apo(a), derived from Lp(a). Also disclosed are methods for determining elastase activity and methods for screening for inhibitors of elastase activity. Methods are also disclosed for purifying, quantitating, and reconstituting active lipoprotein(a), Lp(a).

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

DESCRIPTION

ISOLATION OF apo(a), COMPOSITIONS, AND METHODS OF USE

BACKGROUND OF THE INVENTION

The government has certain right to the present invention to Grant HL-18577 from the National Institutes of Health.

A. FIELD OF THE INVENTION

The present invention relates generally to the field of molecular biology. More particularly, certain embodiments concern methods and compositions comprising purified active apo(a) isoforms, apoB100, and reconstituted lipoprotein(a) [Lp(a)]. In certain embodiments, the invention concerns the use of apo(a) in diagnostic kits in the determination and diagnosis of cardiovascular diseases and lipoprotein disorders. Methods are disclosed for purifying and quantitating apo(a) isoforms, as well as quantitating and reconstituting active Lp(a) complexes.

B. DESCRIPTION OF THE RELATED ART

1. Lipoproteins

Insoluble lipids are transported in the plasma as soluble lipid particles that comprises a core of hydrophobic cholesteryl esters and triglycerides surrounded by a surface monolayer of amphipathic phospholipids with which free cholesterol and apolipoproteins are associated. Apolipoproteins serve as cofactors for enzymes of lipid metabolism and as recognition factors that allow the secretion and uptake of

lipoproteins. Lipoproteins are characterized by their relative density which in turn is determined by the relative amounts of lipid and protein (Havel and Kane, 1989).

Very low density lipoproteins (VLDL) and chylomicrons are triglyceride-rich lipoproteins. VLDL arise from the liver and chylomicrons are from the gut, as they enter the plasma the triglyceride core is hydrolyzed, leaving triglyceride depleted chylomicrons and VLDL. The chylomicrons are taken up by the liver but a large number of the VLDL are further modified in the plasma through the action of lipases and cholesterol ester transferase to yield LDL. High density lipoproteins are generated from excess surface components of VLDL and chylomicrons during the hydrolysis of the triglycerides.

There are two types of apolipoproteins involved in the assembly of lipoprotein: those that remain an integral part of the lipoprotein (a) and those that are more loosely associated and can exchange between particles. Apolipoprotein B100 (ApoB100) is found in the liver and is an integral protein of lipoprotein, its intestinal counterpart is apoB48.

The readily exchangeable lipoproteins include the soluble apolipoproteins. These are gradually lost during triglyceride hydrolysis until LDL contains only apoB proteins. The soluble apolipoproteins are part of a multigene family.

Lipoprotein (a) (Lp(a)) is composed of apo (a) associated through a disulfide linkage to apoB100. High concentrations of Lp(a) in the plasma are a major determinant of coronary heart disease as discussed later. The predicted structure of apo (a) shows homology with plasminogen. The apo (a) component is highly polymorphic leading to a huge variability in Lp(a) structure. The concentration of Lp(a) in the plasma may vary greatly. One of the determinants for this variability seems to be an inverse relationship between the kringle IV domain, and the amount of Lp(a) concentration. Apo (a) is synthesized in the liver of man and other primates.

2. Lp(a) Assembly

Several studies have addressed the complex problem of Lp(a) assembly both in terms of site of occurrence and mechanisms (Ernst *et al.*, 1995; Frank *et al.*, 1994; Frank *et al.*, 1994; Trieu and McConathy, 1995; Chiesa *et al.*, 1992; Brunner *et al.*, 1993; Trieu *et al.*, 1995; White and Lanford, 1994; Edelstein *et al.*, 1994; Tomlinson *et al.*, 1989). Though significant progress has been made, however, many of the issues still remain unresolved. Lp(a) assembly was first shown to occur in the plasma of mice transgenic for human apo(a) given intravenously human LDL (Chiesa *et al.*, 1992). By gel electrophoretic analyses the reassembled Lp(a) was reported to have, like native Lp(a), a disulfide linkage between apo(a) and apoB100. In turn, mouse LDL was considered incompetent to sustain Lp(a) formation with human apo(a). A more direct contribution to the Lp(a) assembly problem has recently come from the studies of Brunner *et al.* (1993), Ernst *et al.* (1995), and Frank *et al.* (1994) using recombinant apo(a) species expressed in various cell systems.

Such studies have led to the proposition that Lp(a) assembly may occur extracellularly representing a two-step process, the first involving non-covalent interactions and the second the formation of a disulfide bond between apo(a) and apoB100. A similar proposal has been originated from earlier studies (Trieu *et al.*, 1995) which used apo(a) recombinant products. Those authors described a 14-residue peptide which they believe may mimic a region in apoB100 potentially involved in the first step of association between this protein and apo(a). No clues on the reassembly process have derived from the study of truncated forms of apoB100 (McCormick *et al.*, 1994; Gabel *et al.*, 1994). In terms of site of assembly, the studies of White and Lanford (1994) in a baboon primary hepatocyte culture system have led the authors to suggest that the association between apo(a) and apoB100 in Lp(a) assembly occurs at the level of the hepatocyte membrane.

The inventors have previously performed studies in human primary hepatocyte cultures (Edelstein *et al.*, 1994) which identified both intracellularly and in the cell

medium, VLDL-like particles having as their protein moiety apoB100 covalently linked to apo(a). The abundance of these particles increased after feeding the cells with oleate bound to albumin. The issue of Lp(a) assembly is clearly a complex issue, which is clouded by uncertainties and discrepancies. The confounding factors might relate to the different models studied and the difficulty of extrapolating them to human biology. Even in the case of the Lp(a) of rhesus monkeys, a species phylogenetically close to man, Tomlinson *et al.* (1989) have shown that the structure of apo(a) of that animal species differs significantly from that of man, *e.g.*, differences in the sequence of the protease domain and absence of kringle V in rhesus. The same may apply to the baboon model.

In this context, studies by the inventors have previously shown that important functional (lysine and fibrinogen binding) differences exist between rhesus and human apo(a) particularly with reference to apo(a) kringle IV-10.

3. Role of Lp(a) in Cardiovascular Disease

The role of Lp(a) as a risk factor for cardiovascular disease is well-documented in a number of prospective and retrospective epidemiological studies (Albers and Marcovina, 1994; Scanu, 1993; Scanu *et al.*, 1991; Utermann, 1995). Unfortunately, limited knowledge exists of the mechanism(s) responsible for the role of Lp(a) in cardiovascular pathogenicity. On structural considerations, Lp(a) may be atherogenic, thrombogenic or both. Some evidence also has been obtained for a function of Lp(a) as a regulator of smooth muscle cell migration and proliferation (Grainger *et al.*, 1993). A potential effect of apo(a) polymorphism on cardiovascular risk has also been suggested but yet to be clearly documented (Scanu, 1993; Scanu *et al.*, 1991; Utermann, 1995). Another important issue yet unresolved is whether the pathogenicity of Lp(a) depends on Lp(a) as a particle and/or apo(a) in its free form.

4. Lysine binding and Lp(a)

In the past it has been suggested that lysine binding may be related to fibrin binding and that Lp(a), by inhibiting plasmin generation, may contribute to the process of atherosclerosis (Loscalzo *et al.*, 1990). It also has been shown that apo(a) kringle IV-10 plays a dominant role in lysine binding through the lysine binding site, LBS.

The presence of a defective LBS activity in kringle IV-10 does not abrogate a lysine-dependent fibrin binding of Lp(a) *via* another apo(a) kringle domain. This implies that a study of this "other" site or sites may prove of importance in evaluating the cardiovascular pathogenicity of Lp(a). This is particularly true for some of those cases seen, which have an Lp(a) that binds poorly to lysine-Sepharose™ (a measure of kringle IV-10 LBS [activity]) and yet present with severe atherosclerotic cardiovascular disease even in the absence of an obvious lipoprotein disorder. In these subjects, the study of the genomic DNA coding for the region between kringle IV-4 and IV-9, the presumed domain for fibrin binding, should prove productive, also in terms of providing the technology for identifying possible functional mutants with the capacity to either increase or decrease the binding of apo(a) to fibrin.

5. Effects of apo(a) on Cardiovascular Pathogenicity

Studies in mice have shown that human free apo(a) injected intravenously is cleared from the circulation more rapidly than Lp(a). This finding suggests that promoting dissociation of apo(a) from Lp(a) may be beneficial in the treatment and/or prevention of cardiovascular disease, since a rapid apo(a) clearance would be associated with a decrease in the plasma Lp(a) levels.

While it may be argued that apo(a), once freed from its association with apoB100, may acquire an athero-thrombogenic potential (perhaps through an uptake by macrophages), no significant studies to date have confirmed that hypothesis. Lawn *et al.* (1992) have reported that mice transgenic for human apo(a) when fed a high fat diet, become more susceptible to atherosclerosis as compared to their non-transgenic littermates. Apo(a) in its free form is potentially more reactive than the apo(a) bound to apoB100-containing lipoproteins.

Unfortunately, the lack of purified active apo(a) in quantity has prevented the assessment of this factor in cardiovascular disease (Albers and Marcovina, 1994). Moreover, the lack of purified apo(a) has prevented the preparation of apo(a)-containing diagnostic reagents for analysis of clinical samples, and thwarted efforts to optimize isolation and purification methods aimed at producing active apo(a). The development of methods to obtain a free apo(a) is imperative for functional and metabolic studies in both animal models and in developing human therapeutics. This need is particularly critical, since a dissociation between apo(a) and LDL may occur at tissue sites, despite the fact that Lp(a) is the more prevalent species in circulating plasma. In fact, it is known that apo(a) free or bound to components of the extracellular matrix, can be present in atherosclerotic areas of the arterial wall (Rath *et al.*, 1989; Beisiegel, 1991).

6. Deficiencies in the Prior Art

The yields of nascent apo(a) isoforms from the media of human primary hepatocyte cultures under are too low to permit obtaining a substantial quantity of native apo(a). Because apo(a) is known to vary in size from individual to individual (M_r = 300,000 to 800,000) a number of isoforms of the protein exist. Moreover, because apo(a) is chemically attached to apoB100, dissociation of apo(a) has been impossible without denaturing and deactivating the protein. Therefore, the development of a method which would permit recovery of large quantities of

dissociated active apo(a) would be a major breakthrough in the determination of the pathogenicity of Lp(a), and provide new tools for therapeutics in the field of cardiovascular medicine. Likewise, the availability of purified apo(a) would be useful in the reconstitution of Lp(a)'s comprising different apo(a) isoforms, and would therefore result in large quantities of purified Lp(a). Moreover, the development of methods of quantitating apo(a), Lp(a-) and native or reconstituted Lp(a) would permit the preparation of diagnostic and therapeutic kits which would find significant utility in the field of cardiovascular medicine. Finally, the availability of purified Lp(a) components would facilitate methods of measuring and reconstituting bioactive Lp(a) complexes.

SUMMARY OF THE INVENTION

To overcome these and other deficiencies in the prior art the present invention provides novel methods and compositions for the preparation of "native" active apo(a) isoforms dissociated from Lp(a)'s using mild reductive conditions which cause the cleavage of the interchain disulfide between apo(a) and apoB100 without an effect on the intrachain disulfides of the individual kringles.

The present invention provides methods for the purification of apolipoprotein (a). This generally comprises providing a composition of lipoprotein (a), contacting said lipoprotein (a) composition with a reducing agent, further contacting said lipoprotein (a) with a lysine analog to produce a reaction mixture containing lipoprotein (a), reducing agent, and lysine analog, incubating said mixture under conditions whereby LDL, unreacted lipoprotein (a) and free apolipoprotein (a) are produced; and separating apolipoprotein (a) from said mixture.

The reducing agents used in the present invention may be one of any reducing agent capable of reducing the disulfide bond of Lp(a) to produce an active apo (a) fraction. Preferably such a reducing agent is homocysteine, N-acetyl cysteine, 2-

mercaptoethanol, 3-mercaptopropionate, 2-aminoethanol, dithiothreitol or dithioerythritol.

In preferred embodiments of the present invention, the reducing agent is dithioerythritol at a concentration between about 0.5mM and 2.0mM.

The lysine analogues are used in the present invention in concentrations sufficient to prevent non-covalent lysine mediated interactions between apo (a) and apoB100, but insufficient to allow precipitation of said proteins from solution. Examples of lysine analogues used in the present invention may include trans 4(amino-methyl)-cyclohexanecarboxylic acid, N-acetyl-L-lysine, p-benzylamine sulfonic acid, hexylamine, benzamidine, benzylamine, L-proline and ε-aminocaproic acid (EACA). In the most preferred embodiments of the present invention the lysine analogue used was EACA in a final concentration ranging between 50mM and 200mM and most preferably between 100mM and 150mM final concentration.

The separation of apo (a) from the rest of the reaction milieu may be achieved by any conventional separation techniques well known to those of skill in the art. Such separation methods include affinity chromatography, ion exchange chromatography, high performance liquid chromatography, ultracentrifugation, and density flotational ultracentrifugation. In the most preferred embodiments of the present invention, the apo (a) is separated using density ultrcentrifugational flotation at 1.21g/ml for 20 hours. Of course, these are only exemplary centrifugational parameters that may be adapted by any practitioner skilled in the art. Because the molecular weight of apo(a) varies from individual to individual (on the order of M_r = 300 to M_r= 800,000) the purification methods disclosed herein provide means for isolating in large quantity intact, active apo(a) from a variety of sources. Reconstitution of these apo(a)'s with isolated apoB100 would therefore provide the basis for the preparation of specific Lp(a) formulations of various molecular weights. This represents a significant advancement over the prior art, since it was not previously possible to

isolate the various apo(a) isoforms from mature Lp(a) without denaturing and inactivating those apo(a) isoforms.

The present invention further describes a method for the purification of fragments of apolipoprotein (a) comprising the steps of providing a composition comprising lipoprotein (a) contacting said lipoprotein (a) composition with a reducing agent; further contacting said lipoprotein (a) composition with a lysine analog to produce a reaction mixture containing lipoprotein (a), reducing agent, and lysine analog; incubating said mixture under conditions whereby LDL, unreacted lipoprotein (a) and free apolipoprotein (a) are produced; separating apolipoprotein (a) from said mixture; contacting said apolipoprotein (a) with a concentration of a proteolytic enzyme whereby fragments of apolipoprotein (a) are produced; and separating said fragments.

In certain embodiments of the present invention the proteolytic enzyme cleaves the bond between Ile3520-Leu3521 of apolipoprotein (a) to produce an F1 fragment and an F2 fragment. The preferred proteolytic enzyme to be used in the present invention is elastase. In preferred embodiments the ratio of apo (a): elastase is 25:1. However, it is understood that those of skill in the art will be able to vary this ratio in the practice of the present invention.

The F1 fragment produced in the present invention has an apparent molecular weight of about 220kDa and said molecular weight varies with size of the phenotype. The F2 fragment produced has an apparent molecular weight of about 170kDa and does not vary appreciably with phenotype. The F2 fragment of the present invention may be further characterized as possessing binding sites for fibrinogen and fibronectin.

The present invention further comprises a method of screening for elastase activity in diseased tissue comprising contacting tissue with a composition comprising apolipoprotein (a); determining the presence of fragments of apolipoprotein a, wherein

the presence of fragments is indicative of the presence of elastase activity in the diseased state. In preferred embodiments of the present invention, the fragments indicative of a diseased state comprise either an F1 fragment, an F2 fragment or a mixture thereof. In further preferred embodiments F1 fragment have an apparent molecular weight of about 220kDa wherein the molecular weight varies according to the size of the phenotype. In other embodiments F2 fragments fragment have an apparent molecular weight of about 170kDa.

Certain embodiments of the present invention disclose a method of screening for inhibitors of elastase activity comprising the steps of obtaining apolipoprotein (a); contacting said apolipoprotein (a) with elastase and a candidate substance for the inhibition of elastase activity; and comparing the cleavage products of apolipoprotein (a) in the presence of the candidate substance with the cleavage products in the absence of the candidate substance whereby the lack of fragments F1 and F2 in the presence of the candidate substance is indicative of inhibition of elastase activity.

In other embodiments the present invention discloses an apo (a) formed according to a method comprising the steps of providing a composition comprising lipoprotein (a); contacting said lipoprotein (a) composition with a reducing agent; further contacting said lipoprotein (a) composition with a lysine analog to produce a reaction mixture containing lipoprotein (a), reducing agent, and lysine analog; incubating said reaction mixture under conditions whereby LDL, unreacted lipoprotein (a) and free apolipoprotein (a) are produced.

In further embodiments, the present invention discloses fragments of apolipoprotein (a) formed according to a method comprising the steps of providing a composition comprising lipoprotein (a); contacting said lipoprotein (a) composition with a reducing agent; further contacting said lipoprotein (a) composition with a lysine analog to produce a reaction mixture containing lipoprotein (a), reducing agent, and lysine analog; incubating said reaction mixture under conditions whereby LDL, unreacted lipoprotein (a) and free apolipoprotein (a) are produced; separating

apolipoprotein (a) from said mixture; contacting said apolipoprotein (a) with a concentration of a proteolytic enzyme to produce fragments of apolipoprotein (a).

The present invention also relates to methods of identifying and quantitating apo(a) and Lp(a), F1 fragments, F2 fragments, or variants thereof. In this aspect, the present invention contemplates diagnostic kits for screening agents or determination of apo(a) isoforms, Lp(a) or apoB100. Said kits can contain active polypeptides of the invention. These kits can contain reagents for detecting an interaction between an agent, or antibody and one of the purified peptides of the present invention, such as apo(a). The provided reagent can be radio-, fluorescence- or enzyme-labeled. The kits can contain a known radiolabeled agent capable of binding or interacting with one of the purified peptides of the present invention, such as purified apo(a). As a specific example, an antibody capable of detecting an apo(a) or an apoB100 complex can be provided. As another specific example, an antibody capable of detecting a specific apo(a) isoform or an apoB100-protein/lipoprotein complex can be provided. In still further examples an antibody capable of detecting an F1 or an F2 fragment can be provided. Reagents for the detection of the lipoprotein complex can be provided. For example, if the compound provided is Lp(a), reagents for detecting the activity of total native or reconstituted Lp(a) (or its particular apo(a) isoform) can be provided.

In another aspect, the present invention contemplates a diagnostic kit for detecting Lp(a) or specific components of Lp(a) such as apo(a) or apoB100. The kit can contain a polynucleotide probe or alternatively an antibody immunoreactive with a Lp(a) or one of its components such as apo(a) or apoB100.

In still further embodiments, the present invention concerns immunodetection methods and associated kits. It is proposed that the apo(a) isoforms or apoB100 peptides of the present invention may be employed to detect antibodies having reactivity therewith, or, alternatively, antibodies prepared in accordance with the present invention, may be employed to detect apo(a), apoB100 or apo(a)/ apoB100-related epitope-containing peptides. In general, these methods will include first

obtaining a sample suspected of containing such a protein, peptide or antibody, contacting the sample with an antibody or peptide in accordance with the present invention, as the case may be, under conditions effective to allow the formation of an immunocomplex, and then detecting the presence of the immunocomplex.

In general, the detection of immunocomplex formation is quite well known in the art and may be achieved through the application of numerous approaches. For example, the present invention contemplates the application of ELISA, RIA, immunoblot (*e.g.*, dot blot), indirect immunofluorescence techniques and the like. Generally, immunocomplex formation will be detected through the use of a label, such as a radiolabel or an enzyme tag (such as alkaline phosphatase, horseradish peroxidase, or the like). Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

For diagnostic purposes, it is proposed that virtually any sample suspected of comprising either an apo(a) or apoB100 peptide or an apo(a)/apoB100-related peptide or antibody sought to be detected, as the case may be, may be employed. It is contemplated that such embodiments may have application in the titering of antigen or antibody samples, in the selection of hybridomas, and the like.

Another aspect of the present invention includes novel compositions comprising isolated and purified active apo(a) isoforms and/or apoB100 proteins, native and reconstituted Lp(a)s F1 fragments, F2 fragments, as well as native and reconstituted Lp(a) complexes. Pharmaceutical compositions prepared in accordance with the present invention find use in a variety of diagnostic and assay kits, as well as providing sources of both active purified apo(a) isoforms and native or reconstituted Lp(a)'s derived from such apo(a) isoforms. Such compositions may be used in the production of antibodies to particular apo(a) isoforms and/or apoB100.

Such methods generally involve administering to an animal a pharmaceutical composition comprising an immunologically effective amount of an apo(a) isoform or apoB100 composition. This composition may include an immunologically-effective amount of either an apo(a) or apoB100 peptide or an apo(a) or apoB100-encoding nucleic acid composition. Such compositions may also be used to generate an immune response in an animal.

In related embodiments, the present invention contemplates the preparation of diagnostic kits that may be employed to detect and/or quantitate the presence of apo(a) isoforms or apo(a)-related proteins or peptides and/or antibodies in a sample. Generally speaking, kits in accordance with the present invention will include a suitable apo(a) protein or peptide or antibody directed against such a protein or peptide, together with an immunodetection reagent and a means for containing the antibody or antigen and reagent. The components of the diagnostic kits may be packaged either in aqueous media or in lyophilized form.

The immunodetection reagent will typically comprise a label associated with the antibody or antigen, or associated with a secondary binding ligand. Exemplary ligands might include a secondary antibody directed against the first antibody or antigen or a biotin or avidin (or streptavidin) ligand having an associated label. Of course, as noted above, a number of exemplary labels are known in the art and all such labels may be employed in connection with the present invention. The kits may contain antibody-label conjugates either in fully conjugated form, in the form of intermediates, or as separate moieties to be conjugated by the user of the kit.

The reagent(s) of the kit can be provided as a liquid solution, attached to a solid support or as a dried powder. Preferably, when the reagent is provided in a liquid solution, the liquid solution is an aqueous solution. Preferably, when the reagent provided is attached to a solid support, the solid support can be chromatograph media, a test plate having a plurality of wells, or a microscope slide.

When the reagent provided is a dry powder, the powder can be reconstituted by the addition of a suitable solvent, that may be provided.

The container means will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the antigen or antibody may be placed, and preferably suitably aliquoted. Where a second binding ligand is provided, the kit will also generally contain a second vial or other container into which this ligand or antibody may be placed. The kits of the present invention will also typically include a means for containing the antibody, antigen, and reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

BRIEF DESCRIPTION OF THE DRAWINGS

The drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Far-ultraviolet circular dichroic spectra of apo(a), parent Lp(a) and RLp(a). Free apo(a), (---); parent Lp(a), (——) isolated from subject K.B. containing the 289 kDa apo(a) isoform; RLp(a), (- - - -) containing the 289 kDa apo(a) isoform and LDL from subject K.B. All samples were equilibrated in 10 mM phosphate buffer, pH 7.5, before the spectra were recorded at 25°C.

FIG. 2A, **FIG. 2B** and **FIG. 2C** depict Lys-Sepharose™ affinity chromatography of human wild-type and rhesus Lp(a) and their respective apo(a)s isolated from Lp(a) by reduction with 2 mM DTE. The dissociated apo(a), 0.3 mg, was isolated by sedimentation at d = 1.127 g/mL. **FIG. 2A** shows human Lp(a) from subject P.T. **FIG. 2B** depicts Rhesus Lp(a) and **FIG. 2C** shows human and rhesus apo(a) chromatographed separately on the same column but displayed here on the one graph.

All samples were dialyzed against Buffer A and loaded onto 2 mL columns. The sample was applied at a flow rate of 7.8 mL/h. After washing with four column volumes of PBS, the bound components were eluted with 200 mM EACA at a flow rate of 15 mL/h.

FIG. 3A and **FIG. 3B** illustrate the effect of time of incubation on the reassembly of Lp(a) from apo(a) and LDL. **FIG. 3A** shows apo(a) from subject B.K. (•-•) or from rhesus (m-m) was incubated with LDL (subject B.K.) at an apoB100:apo(a) molar ratio of 50:1 for the designated time intervals at 37°C. The reassembled products were separated by flotation at d = 1.127 g/mL and quantitated by ELISA. **FIG. 3B** shows apo(a) from subject B.K. incubated with VLDL (§-§) from a dyslipidemic subject under the same conditions. The results are presented as the percent of the total apo(a) mass in the reaction mixture that underwent reassembly.

FIG. 4A, **FIG. 4B** and **FIG. 4C** illustrate size distribution data obtained from electron micrographs of human Lp(a), RLp(a) and LDL. All samples were obtained from subject K.B. with the 289 kDa apo(a) phenotype at a concentration of 50 mg/mL in 10 mM NH_4HCO_3. **FIG. 4A** shows control Lp(a). The electron micrograph was taken at an instrumental magnification of 46,000. The bar graph shows the size distribution of particles. **FIG. 4B** shows RLp(a) isolated by lysine-Sepharose™ chromatography. The electron micrograph was taken at an instrumental magnification of 46,000. The bar graph shows the size distribution of particles. **FIG. 4C** illustrates control LDL. The electron micrograph was taken at an instrumental magnification of 46,000. The bar graph shows the size distribution of particles.

FIG. 5. Models of Lp(a) assembly for Trp72 and Arg72 apo(a). Kringles IV-4 to 10 and kringle V representing the COOH terminal domain of apo(a) are drawn as circles and the protease region (P) as a rectangle. The interchain disulfide between kringle IV-9 and apoB100 is depicted as present in both the wild-type and mutant apo(a). When the disulfide bridge is intact, lysine binding of Lp(a) occurs *via* the LBS of kringle IV-10. Once the disulfide is cleaved by the action of DTE, the apo(a)

domain between kringles IV-4 and 10 interacts with proline and lysine, a property not exhibited by apo(a) bound to apoB100.

FIG. 6. Fractionation by lysine-Sepharose™ of the products from the limited digestion of apo(a) with pancreatic elastase. The apo(a) digest was dialyzed against buffer A before loading onto a 7 ml column. The sample was applied at a flow rate of 7.8 ml/h. After washing with three column volumes of PBS, the column was further washed with 500 mM NaCl to elute non-specifically bound material; thereafter, the bound component was eluted with 200 mM EACA at a flow rate of 15 ml/h.

FIG. 7. Alignment of the partial amino acid sequences of F1 and F2. F1 and F2 were purified from the elastase digests of Lp(a) and apo(a) as outlined in the text and prepared for microsequencing as described. Apo(a) is composed of repeats of KIV numbered 1-10, one KV and a protease domain (P) according to the nomenclature of Scanu and Edelstein (1995). The first 20 amino acids obtained by sequencing F1 (SEQ ID NO:8) were aligned to the amino acid sequence of the mature apo(a) without the signal peptide sequence (SEQ ID NO:7). The amino acid sequence of F2 began with leucine at position 3521 (SEE SEQ ID NO:9). The elastase cleavage site was determined to be in the linker region between KIV-4 and 5 (SEQ ID NO:10). The dashed lines refer to undetermined amino acids and the dotted lines are continuation of sequences upstream and downstream of the protein sequences. Alignments to the deduced amino acid sequence of apo(a) (McLean *et al.*, 1987) were performed with the ClustalW sequence alignment program.

FIG. 8. Schematic diagrams of the structure of Lp(a), R-miniLp(a), apo(a), F1 and F2. These structures are drawn to emphasize the kringles and are not meant to be actual depictions of Lp(a) or apo(a) structure. KIV-9 contains the single sulfhydryl which is in covalent linkage to apoB100 of LDL.

FIG. 9A, FIG. 9B and FIG. 9C. Bar graphs of the size distribution of particles in electron micrographs of human Lp(a) (FIG. 9A), miniLp(a) (FIG. 9B)and

R-miniLp(a) (FIG. 9C). All samples were at a concentration of 50 μg/ml in 10 mM NH_4HCO_3.

FIG. 10A and FIG. 10B. Binding of R-miniLp(a) and apo(a) fragments to fibrinogen and fibronectin. R–miniLp(a) (o), F2 (•) and F1 (■), at concentrations in the range of 0 and 125 mM, were incubated for 2 h at 22°C with either immobilized fibrinogen (**FIG. 10A**) or fibronectin (**FIG. 10B**) as described in the Examples. The binding to fibrinogen (FIG. 10A) was expressed as a specific lysine-mediated binding obtained by subtracting the binding in the presence of 200 mM EACA from the total binding. The binding to fibronectin (**FIG. 10B**) is represented by the total binding due to the absence of an inhibitory effect of EACA. The data are the means of two determinations for a representative study.

FIG. 11. Clearance of Lp(a) and derivatives from mouse plasma: Mice were injected with 25-250 μg of R-miniLp(a) (■, closed squares), Lp(a) (□, open squares), F2 (Δ, open triangles), apo(a) (•, closed circles), unfractionated apo(a) digest (o, open circles) and F1 (▼, closed inverted triangles) in sterile PBS. Blood samples (100 μl) were collected from the orbital vein in heparinized haematocrit tubes and plasma apo(a) immunoreactive components were measured by ELISA as described in the Examples.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The pathogenicity of Lp(a) has been documented in a number of studies. It is unknown whether the pathogenicity arises from the Lp(a) molecule *per se* and/or from apo (a) in its free from. A major drawback in answering these questions is the lack of readily available, purified apo (a) of known size. The present invention provides methods and compositions for the preparation of "native" active apo(a) isoforms dissociated from Lp(a)'s using mild reductive conditions which cause the cleavage of the interchain disulfide between apo(a) and apoB100 without an effect on the

intrachain disulfides of the individual kringles. The present invention further provides convenient methods for the screening of inhibitors of elastase activity.

A. PURIFICATION OF APO (A)

The purification of apo (a) involves obtaining a composition of Lp(a) and subjecting the composition to reductive cleavage in a manner that allows the formation of cleavage products apo (a) and apoB100. These products are then separated to yield purified apo (a).

1. Lipoprotein(a)

Lipoprotein(a), Lp(a), refers to a class of lipoprotein particles in which apoB100 is covalently linked to apolipoprotein(a), apo(a), by a disulfide bond (Koschinsky *et al.*, 1993; Brunner *et al.*, 1993; Van Der Hoek *et al.*, 1994; Scanu and Fless, 1990). Apo(a) is a large glycoprotein, polymorphic in size (250-800 kDa), containing kringles highly homologous to kringles 4 and 5 of plasminogen (Scanu and Edelstein, 1995). Some of the kringles in apo(a) have been predicted to have a functional lysine binding site (LBS), in particular kringle IV-10, comprising two anionic, Asp55 and Asp57, two cationic, Arg35 and Arg71, and three non-polar, Trp62, Trp72 and Phe64 amino acids (Guevara *et al.*, 1992, 1993). LBS is also present in plasminogen kringle 4 except that Lys35 has been replaced by Arg. Studies on apo(a) kringle IV-10 cloned and expressed in *Escherichia coli*, showed that this kringle binds avidly to lysine and its analogues (LoGrasso *et al.*, 1994). Lp(a) has been assayed using a variety of immunological methods, with these studies indicating an elevated Lp(a) level was associated with an increased risk of cardiovascular and cerebrovascular disease (Albers and Marcovina, 1994).

The information regarding the site(s) of the Lp(a) assembly process remains unclear. An intracellular assembly has been observed in primary human hepatocyte

cultures (Edelstein *et al.*, 1994) whereas an extracellular event has been reported in cultures of baboon hepatocytes (White *et al.*, 1993; White and Lanford, 1994), HepG2 cells (Koschinsky *et al.*, 1991) and by infusing human low density lipoprotein, (LDL) into mice transgenic for a human 17 kringle apo(a) construct (Chiesa *et al.*, 1992). The assembly of Lp(a) was also shown to occur in mice transgenic for human apoB100 and a 17 kringle apo(a) construct (Linton *et al.*, 1993; Callow *et al.*, 1994). In all cases, the reassembled lipoprotein was reported to have an apo(a) disulfide linked to apoB100. In fact, the requirement for this disulfide has been documented by Brunner *et al.*, (1993).

2. Purification of Lp(a)

Lp(a) is known to be made in the liver of primates. The inventors' present study also indicates that Lp(a) may be present in other species. The LDL and VLDL in the plasma represents the primary source for the purification of Lp(a). Plasma may be collected from any primate source for the purposes of the invention, or indeed any other source suspected of possessing Lp(a). The Lp(a) component of the plasma can then be separated from other components of the plasma using ultracentrifugational flotation at a density of 1.21 g/mL for 20 hours at 50, 000rpm followed by affinity chromatography using lysine-Sepharose™. Of course, the ultra centrifugational procedure is only exemplary and those of skill in the art will be able to vary them according to the particular equipment and study need without undue experimentation. The plasma may be supplemented with various inhibitors, for example, EACA and proline to prevent the Lp(a) from interacting with LDL components of the plasma.

Having separated Lp(a) from the other plasma components the Lp(a) sample is purified using affinity chromatography lysine-Sepharose™ chromatography. In this step, columns are packed in a ratio of, for example 5:1 of lysine-Sepharose™:Lp(a) protein. Of course this ratio may vary according to the size of the column, the elution conditions to be used and the amounts of Lp(a) needing to be purified. The columns are equilibrated with an appropriate buffer containing for example, 0.02%NaN_3. NaN_3 is a bacteriostatic agent and may be substituted with any other suitable agent known to

those of skill in the art. Lp(a) fractions eluted from the lysine-Sepharose™ columns are dialyzed to remove excess salts in a suitable buffer containing 0.02% NaN3 at a pH of 7.5.

In some cases, it is desirable to you a method other than lysine-Sepharose™ chromatography for the purification of Lp(a), in such instances other chromatographic methods for example FPLC may be employed. Such methods are disclosed in Scanu *et al.*, 1993, incorporated herein by reference, and may be used in conjunction with the present invention to purify apo (a) from Lp(a).

The product purity can be assessed by for example, mobility on, 1% agarose gels, Western blots of SDS PAGE, utilizing anti-Lp(a) antibodies.

One of the problems with Lp(a) isolated from some natural sources is low yields. The present invention also discloses methods for enhanced production of Lp(a) by recombinant methodologies in a suitable eukaryotic or bacterial hosts, employing DNA constructs to transform cell lines, yeast cells, or Gram-positive or Gram-negative bacterial cells can also be used. For example, the use of *Escherichia coli* expression systems are well known to those of skill in the art, as is the use of other bacterial species such as *Bacillus subtilis* or *Streptococcus sanguis.*

Further aspects of the invention include high level expression vectors incorporating DNA encoding the novel genes encoding components of Lp(a) and particular variants thereof. It is contemplated that vectors providing enhanced expression of Lp(a) components in other systems such as *S. mutans* will also be obtainable. Where it is desirable, modifications of the physical properties of Lp(a) components may be sought to increase its solubility or expression in liquid culture. The genes may be placed under control of a high expression promoter or the components of the expression system altered to enhance expression.

3. Isolation of Apo (a) from Lp (a)

(a) Apo (a) isolation from Lp(a) using centrifugation

Following the purification of Lp(a), the novel methods of the present invention are employed to generate dissociated active apo (a) from the Lp(a) complex. The purified intact Lp(a) protein is subjected to mild reductive cleavage wherein a known volume of Lp(a) of a suitable concentration for example, 1mg/ml in buffer of pH 7.5 is incubated with a reductant such as DTE at a final concentration of 1.5-2mM.

The reaction is incubated at room temperature for 10-20 minutes. This is followed by an incremental addition of ε-aminocaproic acid (EACA) to a final concentration of 100-150 mM. EACA is used in concentrations sufficient to prevent non-covalent, lysine mediated interactions between apo (a) and apoB100. EACA may be substituted by other lysine analogues, for example, compounds such as trans 4(amino-methyl)-cyclohexanecarboxylic acid, N-acetyl-L-lysine, p-benzylamine sulfonic acid, hexylamine, benzamidine, benzylamine, L-proline. Of course these are only exemplary lysine analogues and those of skill in the art may use other lysine analogues to prevent interaction between apo (a) and apoB100 proteins.

The reaction mixture is stirred slowly in an anoxic environment and is kept protected from light. The incubation period is for a time sufficient to allow the dissociation of apo (a) and apoB100 from Lp(a), in a preferred embodiment this period is 1 hour, however, it is understood that this time may vary with each experimental procedure. The dissociated Lp(a) components are then dialyzed in a suitable buffer of pH containing 0.02% NaN3 and 100mM EACA. After dialysis the density of the mixture is adjusted with 60% sucrose in the same buffer. The final solution is ultracentrifuged at 2-10°C for a suitable period of time to allow density separation of free apo (a) from LDL and unreacted Lp(a). The purified apo (a) is in the bottom layer of the supernatant. Of course, the conditions for the separation of apo (a) from the reaction mixture using sucrose density ultracentrifugation is only exemplary, and other methods commonly used by those of skill in the art may be used.

The yield of apo (a) from the above process is between 80-100 percent. Of course, this yield may vary according to the particular conditions and reagents used by the practitioner. The methodology described may facilitate the isolation of a variety of various apo(a) isoforms in large quantity. These isoforms can then be separated using chromatographic and other separation techniques to yield highly pure isoforms of active apo (a). These purified apo (a) isoforms can be used in the reassembly of Lp(a) to generate known Lp(a)s of specific size and characteristics to facilitate the study of Lp(a) metabolism and to monitor its pathogenic properties.

(b) Isolation of Apo (A) Using Chromatographic Methods Under Reducing Conditions

As an alternative to the above methods for the isolation of apo (a) from Lp(a) chromatographic methods may be utilized as exemplified below.

Heparin Sepharose™ Chromatography

Lp(a) may be treated with a reducing agent in the presence of a lysine analogue. For the purposes of this invention the lysine analog is supplied to prevent the interaction of apo (a) with apoB100. The reducing agent is supplied to break the disulfide bond of Lp (a). Lysine analogs for this invention include but are not limited to compounds such as EACA, trans 4(amino-methyl)-cyclohexanecarboxylic acid, N-acetyl-L-lysine, p-benzylamine sulfonic acid, hexylamine, benzamidine, benzylamine, L-proline or any other lysine analogue known to the artisan skilled in the art may be used. Example of reducing agents that may be used in this invention include but are not limited to homocysteine, N-acetyl cysteine, 2-mercaptoethanol, 3-mercaptopropionate, 2-aminoethanol, dithiothreitol, and DTE.

In a specific example the mixture of Lp (a), 1-2mM DTE as a reducing agent and 50mM EACA as a lysine analog is incubated for a suitable period of time in a suitable buffer of pH 7.4. A heparin-Sepharose™ column is equilibrated with a suitable buffer containing 50mM EACA and 1mM DTE. The mixture is applied to the

equilibrated column, the column is washed with the same buffer and the first eluate is collected.

The first eluate from the column contains the apo (a) dissociated from Lp (a). The "free" apo (a) is dialyzed against an appropriate buffer. the dialysis product is pure apo (a) that may be freeze dried and stored at -20°C or used immediately. The column is further washed with the buffer for a total of three column volumes followed by 3 volumes of 2M NaCl in the buffer. The high salt concentration serves to dissociate the remaining unreacted Lp(a) and LDL free of apo (a).

Lysine-Sepharose™ Chromatography

An alternative to heparin-Sepharose™ chromatography is lysine chromatography. In this type of separation Lp(a) is treated with a suitable reducing agent for example 1-2mM DTE for a suitable period of time and then applied to a lysine Sepharose™ column that has been equilibrated with a suitable buffer of pH 7.4 containing for example 1mM DTE (elution buffer C). the column is washed with the same buffer and the first volume of elute is collected. This fraction contains LDL dissociated from apo (a). The column is then further washed with the buffer for an appropriate number of column volumes of the elution buffer C, this is followed by a further elution with a similar buffer containing a high concentration of lysine analog for example 200mM EACA in 10mM pH 7.4 phosphate buffer (elution buffer D). Elution with 3-5 column volumes of buffer D will dissociate the remaining Lp (a) and free apo (a). This second eluate containing the free apo (a) can then be centrifuged in 30-50% sucrose containing 100mM EACA by procedures well known to those of skill in the art. The Lp(a) will migrate to the top floating layer and the free apo (a) will be in the sedimenting layer.

B. F1 AND F2 FRAGMENTS FROM APO (A) AND LP(A).

1. Proteolytic Generation of F1 and F2

Lp(a) and apo (a) as purified in the steps above can be subjected to proteolytic cleavage to yield fragments of a known size. These fragments have been characterized as the F1 and F2 fragments from apo (a). Apo (a) is subjected to proteolytic cleavage using elastase an apo (a): elastase ratio of 5:1 to 100:1 can be used for the generation of the proteolytic fragments. In some embodiments a ratio of 50:1 was used in other a ratio of 10:1 was used. In preferred embodiments a ratio of 25:1 apo protein:elastase protein was used. The apo (a) and elastase enzyme were incubated in a suitable buffer containing KI for time period of 1-24 hours at about 18-22°C. In certain embodiments of the invention the time period was between 2 hours and 20 hours in other embodiments of the invention the time period of for incubation varied between 4 hours and 15 hours in other embodiments of the invention the time for incubation was between 5 hours and 10 hours. In preferred embodiments of the present invention the incubation time was 2 hours. Of course it is understood that this time could be greater or less depending on the demands of the particular assay being performed.

After a suitable incubation period the reaction is terminated by the addition of 5mM DFP and a further incubation for a suitable period of time. In the most preferred embodiments this second incubation time is 20 minutes. Of course this time can vary from one study to another. Lp(a) can also be used to generate fragments through elastase mediated cleavage. The F1 and F2 fragments may then be separated according to conventional protein separation methodology well known to those of skill in the art. These methods include but are not limited to HPLC, affinity chromatography, centrifugation, and electrophoresis among others. Ion exchange and affinity chromatography would be useful in the isolation of purified F1 and F2 fragments.

The F2 fragment binds to LDL, lysine-Sepharose, heparin-Sepharose, fibrinogen, and fibronectin. In preferred embodiments columns of lysine-Sepharose,

heparin-Sepharose or of immobilized LDL fibrinogen, and fibronectin are utilized to bind F2 from a mixture of F1 and F2 generated by elastolytic activity. The F1 fraction is recovered in the buffered flow-through volume from these columns. The F2 fragments can then be preferentially eluted from the columns by adjusting the buffer to a high ionic strength, for example 500mM Nacl, and containing a high concentration of lysine analog for example 200mM EACA. The purified F1 and F2 fragments can then be lyophilized and stored at -20°C.

2. Properties of F1 and F2 fragments.

The proteolytic fragments generated in the digestion are then subjected to Western blot analysis and probed with anti apo (a) antibodies. In the case of apo(a), the cleavage by pancreatic elastase at the Ile3520-Leu3521 bond generated two discrete fragments which was called F1 and F2. F1 comprised kringles IV-1, IV-2 repeats, IV-3 and IV-4 and corresponded to the N-terminal domain of apo(a). Consistent with the chemical data was the observation that by electrophoretic criteria the size of F1 varied according to apo(a) isoform size which is dependent on the number of kringle IV-2 repeats (Lackner *et al.*, 1991; McLean *et al.*, 1987). F2 comprised kringles IV-5 to IV-10, kringle V and the protease region and represented the C-terminal domain of apo(a).

As in the case of apo(a), elastase cleavage of Lp(a) produced F1 and F2. However, F2 was linked to LDL in the form of an LDL/F2 complex which was called miniLp(a) because it was smaller than the parent Lp(a) by having only one apo(a) fragment and also by electrophoretic criteria. Huby *et al.* (1995) reported the generation of a miniLp(a) particle by subjecting Lp(a) to limited digestion by thermolysin. This enzyme also cleaved apo(a) in the linker between kringles IV-4 and IV-5 but at the Ala3513-Phe3514 bond which is seven amino acids upstream of the elastase cleavage site. Thus, the miniLp(a) generated by the thermolysin digestion method contains a truncated apo(a) which is 7 amino acids longer than their F2. Unfortunately, as shown by Huby *et al.* (1995) and the current studies, neither

thermolysin- nor elastase-generated miniLp(a) particles are ideal end-products because both enzymes cause a partial proteolysis of apoB100 with retention of the fragments on the lipoprotein particle. According to the results of the current studies, a better way to produce miniLp(a) is by the reassembly approach exploiting the capacity of F2 to covalently associate with LDL. The resulting R-miniLp(a) resembles elastase-derived miniLp(a) but it has the important advantage of containing intact apoB100. For these reasons, R-miniLp(a)) was preferentially used in the inventors' work.

Only F2 and the F2-containing R-miniLp(a) bound to fibrinogen whereas F1 was inactive. The activity of F2 was expected since F2 contains the domain between kringles IV-5 to IV-8 which, as was previously reported (Klezovitch *et al.*, 1996), is involved in the binding of apo(a) to fibrinogen and is partially masked when it is linked to apoB100 (Edelstein *et al.*, 1995; Klezovitch *et al.*, 1996). In the latter respect, the current studies determined that F2, in its free form, had a higher binding capacity for fibrinogen than when it was a member of R-miniLp(a). Moreover, a functional divergence between F2 and F1 was observed in the fibronectin binding studies pointing to the fact that in apo(a), F2 is the fragment which has potentially relevant biological functions. As described below animal studies unveiled further differences in the F1 and F2 fragments.

F1 injected into mice had a short residence time in the plasma and was also rapidly excreted in the urine in the form of several fragments. After injection into the mouse the F1 fraction appears as distinct 4-5 electrophoretic bands in the size range of 220-135 kDa and as markedly smaller ones in the urine (size range 100-33 kDa). These fragments show that elastase activity in the plasma acts on apo (a).

F2 has different retention characteristics to F1 in that there is a longer retention time and relatively small amounts are excreted. In human subjects, fragments of apo(a) are spontaneously present in their plasma. These fragments represent about 5% of the total plasma Lp(a) protein and are significantly larger than

those in the urine. Moreover, the fragments in the urine have the size and band pattern of those seen in the urine of injected mice.

Elastase-dependent apo(a) fragmentation generating F1 and F2 fragments could be occurring under pathological conditions, for instance at sites of inflammation involving an active recruitment of polymorphonuclear cells and macrophages. Of interest, the presence of apo(a) fragments has been reported in human atherosclerotic lesions (Hoff *et al.*, 1994). The present studies show that these fragments would be of the F2-type and thus unaffected by the F1-dependent size polymorphism of apo(a). As a corollary to this, F2 would be more pathogenic than F1 from the cardiovascular standpoint.

C. ROLE OF apo(a) IN Lp(a) ASSEMBLY

Phillips *et al.*, (1993) working with an apo(a) 17 kringle construct, demonstrated an important role for non-covalent interactions in Lp(a) assembly. Several studies (Ernst *et al.*, 1995; Frank *et al.*, 1994a, b; Trieu and McConathy, 1995) have provided evidence that a lysine dependent site(s) in kringle IV-10 and in kringles IV-5 through IV-9 may be important in the early assembly step(s) preceding the stabilization of the Lp(a) complex by a disulfide linkage. Trieu *et al.* (1991) demonstrated that like ε-aminocaproic acid (EACA), proline inhibits the binding of recombinant apo(a) to LDL and concluded that these reagents may bind to the same site on apo(a).

It has been shown (Scanu *et al.*, 1993) that rhesus monkey Lp(a) has an impaired lysine binding capacity due to the presence of Arg72 in the apo(a) kringle IV-10 LBS. Human subjects have been identified in whom the Lp(a) is lysine binding defective (Lys^-) (Scanu *et al.*, 1994) having in the LBS of kringle IV-10, Arg72 instead of the Trp72 present in wild-type positive lysine binding (Lys^+) subjects. The Lys^- human subjects have plasma levels of Lp(a) which are too low to unequivocally define whether they represent either an Lp(a) complex, free apo(a) or both. On the

other hand, the rhesus monkeys, in spite of their Lys^- Lp(a), have plasma levels which are about 3-fold higher than those of control Lys^+ human subjects (Scanu *et al.*, 1993).

The present invention describes a novel method for the disassembly and reassembly of both human and rhesus Lp(a). This technology has been applied to the analysis of some of the determinants of Lp(a) assembly taking advantage of the Lys^+ and Lys^- Lp(a) models.

D. ELASTASE

1. Inhibitors of Elastase Activity

Elastase is serine protease whose normal role is in the phagocytosis and defense against microbial infection (Bode *et al.*, 1989). Elastases are also capable of destroying many connective tissues and elastase activity has been found in numerous diseased states including pulmonary emphysema and rheumatoid arthritis (Tetley, 1993). Natural inhibitors of elastase activity include bovine pancreatic tryptin inhibitor, α_1 proteinase inhibitor, and macroglobulin. In many chronic wounds elastolytic activity goes uncontrolled, because the diseased state results in the inactivation of natural inhibitors of elastase, thereby leading to the elastolytic breakdown of the extracellular matrix (Carrell *et al.*, 1982). This scenario is well demonstrated in cases with bronchoalveolar lavages where it has been shown that there is a reduction in anti-elastase activity in smokers which leads to the autolysis in tissue (Castell *et al.*, 1988). It is, therefore, well recognized by those of skill in the art that generating inhibitors of elastase activity is of the utmost importance (Kraunsoe *et al.*, 1996).

2. Screening for Inhibitors of Elastase Activity

The present invention seeks to overcome drawbacks inherent in the prior art by providing compositions and kits for screening for inhibitors of elastase activity.

The invention provides methods for screening for inhibitors of elastase activity by testing for the presence of F1 and F2 fragments in the absence of the candidate substance and comparing such results to the assay performed in the presence of candidate inhibitors of elastase. The inventors have discovered that the use of elastase on apo (a) and on Lp(a) characteristically yields two fragments of known size and functional characteristics.

In certain embodiments, the present invention concerns a method for identifying further inhibitors. It is contemplated that this screening technique will prove useful in the general identification of any compound that will inhibit elastase activity in cells.

The active compounds may include fragments or parts of naturally-occurring compounds or may be only found as active combinations of known compounds which are otherwise inactive. However, prior to testing of such compounds in humans or animal models, it will possibly be necessary to test a variety of candidates to determine which have potential.

Accordingly, in screening assays to identify pharmaceutical agents which inhibit elastase activity, it is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds.

In these embodiments, the present invention is directed to a method for determining the ability of a candidate substance to inhibit the activity of elastase the method including generally the steps of:

(a) obtaining apo (a);

(b) contacting apo (a) with elastase and a candidate substance for the inhibition of elastase activity; and

(c) determining the ability of the candidate substance to inhibit the elastase activity of the cell by comparing the cleavage products of apo (a) in the presence of the candidate substance, whereby the lack of F1 and F2 fragments in the presence of such a substance is indicative of inhibition of elastase activity.

To identify a candidate substance as being capable of inhibiting elastase activity, one would measure or determine activity in the absence of the added candidate substance by adding apo (a) or Lp(a) and monitoring the generation of F1 and F2 fragments arising as a result of elastase activity. One would then add the candidate substance to the cell and re-determine the levels of the F1 and F2 fragments that arise upon addition of apo (a) or Lp(a) in the presence of the candidate substance. A candidate substance which reduces the elastase activity, and thereby reduces the levels of F1 and F2 generated, relative to the activity in its absence is indicative of a candidate substance with inhibitory capability.

The candidate screening assay is quite simple to set up and perform, and is related in many ways to the assay discussed above for determining elastase activity. Thus, after obtaining elastase, one will admix a candidate substance with the enzyme, under conditions which would allow the formation of F1 and F2 fragments but for inclusion of an inhibitor substance. In this fashion, one can measure the ability of the candidate substance to inhibit the elastase relatively in the presence of the candidate substance. Elastase may be supplied in a pure form or in an endogenous from cellular extracts.

"Effective amounts" in certain circumstances are those amounts effective to reproducibly reduce elastase activity, or to reduce the F1 and F2 fragments, in comparison to their normal levels. Compounds that achieve significant appropriate changes in activity will be used.

Significant decrease in F1 and F2 formation, *e.g.*, as measured using chromatography techniques, are represented by a reduction in F1 and F2 levels of at least about 30%-40%, and most preferably, by increases of at least about 50%, with higher values of course being possible. Assays that measure F1 and F2 content are discussed in the specification.

It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

3. New Elastase Inhibitors

New inhibitors of elastase activity may be used *in vivo* for the treatment of various diseased states. Natural inhibitors of elastase activity, such as A1 proteinase inhibitor and macroglobulin are inactivated in numerous diseased states, thereby leading to uncontrolled elastolytic activity. Inhibitors of elastase activity as isolated according to the present invention may be used inhibit the activity of elastase in such diseased states hence limiting the amount of breakdown of the extracellular matrix. Pharmaceutical compositions comprising such inhibitors can be formulated for the treatment of inflammation or to inhibit the elastolytic breakdown of cellular tissue that occurs as a result of unchecked elastase activity. Methods for *in vitro* and *in situ* delivery protocols of such formulations are well within the grasp of those skilled in the art.

E. PHARMACEUTICAL COMPOSITIONS

The compositions disclosed herein may be used in the preparation of pharmaceutical preparations for administration to an animal. Such administration may be desirable for the induction of an immune response or for diagnosis of a specific disease or disorder. Such administration may be oral, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of the unit. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In

addition, the active compounds may be incorporated into sustained-release preparation and formulations.

The active compounds may also be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients

enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

For oral administration the polypeptides of the present invention may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein (a)s an active ingredient is well understood in the art. Typically, such

compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptablesalts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human

administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

F. MONOCLONAL ANTIBODIES, ELISAs AND WESTERN BLOTS

1. The Generation of Monoclonal Antibodies

In another aspect, the present invention contemplates an antibody that is immunoreactive with apo (a), Lp(a), F1, F2 or any other polypeptide of the invention. An antibody can be a polyclonal or a monoclonal antibody. In a preferred embodiment, an antibody is a monoclonal antibody. Means for preparing and characterizing antibodies are well known in the art (See, *e.g.*, Howell and Lane, 1988).

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster or a guinea pig. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Antibodies, both polyclonal and monoclonal, specific for isoforms of antigen may be prepared using conventional immunization techniques, as will be generally known to those of skill in the art. A composition containing antigenic epitopes of the compounds of the present invention can be used to immunize one or more experimental animals, such as a rabbit or mouse, which will then proceed to produce specific antibodies against the compounds of the present invention. Polyclonal antisera may be obtained, after allowing time for antibody generation, simply by bleeding the animal and preparing serum samples from the whole blood.

To obtain monoclonal antibodies, one would also immunize an experimental animal, often preferably a mouse, with an antigenic composition. One would then, after

a period of time sufficient to allow antibody generation, obtain a population of spleen or lymph cells from the animal. The spleen or lymph cells can then be fused with cell lines, such as human or mouse myeloma strains, to produce antibody-secreting hybridomas. These hybridomas may be isolated to obtain individual clones which can then be screened for production of antibody to the desired peptide.

Following immunization, spleen cells are removed and fused, using a standard fusion protocol with plasmacytoma cells to produce hybridomas secreting monoclonal antibodies against apo(a) related antigen, as used herein apo (a) related antigen or peptide refers to Lp(a), apo (a), F1 fragments of apo (a), F2 fragments of apo (a), or variants thereof. Hybridomas which produce monoclonal antibodies to the selected antigens are identified using standard techniques, such as ELISA and Western blot methods. Hybridoma clones can then be cultured in liquid media and the culture supernatants purified to provide the antigen-specific monoclonal antibodies.

It is proposed that the monoclonal antibodies of the present invention will find useful application in standard immunochemical procedures, such as ELISA and Western blot methods, as well as other procedures which may utilize antibody specific to apo(a) related antigen epitopes.

Additionally, it is proposed that monoclonal antibodies specific to the particular apo(a) isoforms may be utilized in other useful applications. For example, their use in immunoabsorbent protocols may be useful in purifying native or recombinant apo(a) isoforms or variants thereof.

In general, both poly- and monoclonal antibodies against apo (a) related antigens may be used in a variety of embodiments. For example, they may be employed in antibody cloning protocols to obtain cDNAs or genes encoding apo(a) or related proteins. They may also be used in inhibition studies to analyze the effects of apo(a) related peptides in cells or animals. Anti-apo(a) related antigen antibodies will also be useful in immunolocalization studies to analyze the distribution of apo(a) related

peptides during various cellular events, for example, to determine the cellular or tissue-specific distribution of the apo(a) related peptide under different physiological conditions. A particularly useful application of such antibodies is in purifying native or recombinant apo(a) related peptide, for example, using an antibody affinity column. The operation of all such immunological techniques will be known to those of skill in the art in light of the present disclosure.

Means for preparing and characterizing antibodies are well known in the art (See, *e.g.*, Harlow and Lane, 1988; incorporated herein by reference).

The methods for generating monoclonal antibodies (mAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic composition in accordance with the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, *m*-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune

response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster, injection may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate mAbs.

mAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, *e.g.*, a purified or partially purified apo(a) isoform protein, polypeptide or peptide. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B-lymphocytes (B-cells), are selected for use in the mAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells

are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, 1986; Campbell, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 ratio, though the ratio may vary from about 20:1 to about 1:1, respectively, in the

presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described (Kohler and Milstein, 1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.*, (1977). The use of electrically induced fusion methods is also appropriate (Goding, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B-cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B-cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme

immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide mAbs. The cell lines may be exploited for mAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide mAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the mAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. mAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

2. The Use of ELISAs and Western Blots to Screen for Apo (a) and Fragments F1 and F2

ELISAs may also be used in conjunction with the invention. The novel active dissociated apo(a) isoforms may be used in the preparation of specific apo(a) antibodies and the F1 and F2. It is known that Lp(a) is involved in the various cardiovascular diseases. The present invention provides for the preparation of monoclonal antibodies to apo (a), F1 and F2 and also for Lp(a). These antibodies can be used in characterizing the Lp(a) status of healthy and diseased tissues, through techniques such as ELISAs and Western blotting.

The use of anti-Lp(a), anti-apo(a), anti-F1 and anti-F2 antibodies or antibodies to variants thereof, in an ELISA assay is contemplated. Apo(a), F1 and F2, proteins or antigenic sequences derived therefrom are immobilized onto a selected surface,

preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed material, it is desirable to bind or coat the assay plate wells with a nonspecific protein that is known to be antigenically neutral with regard to the test antisera such as bovine serum albumin (BSA), casein or solutions of powdered milk. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

After binding of antigenic material to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the antisera or clinical or biological extract to be tested in a manner conducive to immune complex (antigen/antibody) formation. Such conditions preferably include diluting the antisera with diluents such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween®. These added agents also tend to assist in the reduction of nonspecific background. The layered antisera is then allowed to incubate for from about 2 to about 4 hr, at temperatures preferably on the order of about 25° to about 27°C. Following incubation, the antisera-contacted surface is washed so as to remove non-immunocomplexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween®, or borate buffer.

Following formation of specific immunocomplexes between the test sample and the bound antigen, and subsequent washing, the occurrence and even amount of immunocomplex formation may be determined by subjecting same to a second antibody having specificity for the first. To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the antisera-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (*e.g.*, incubation for 2 hr at room temperature in a PBS-containing solution such as PBS/Tween®).

After incubation with the second enzyme-tagged antibody, and subsequent to washing to remove unbound material, the amount of label is quantified by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline)-6-sulfonicacid (ABTS) and H_2O_2, in the case of peroxidase as the enzyme label. Quantitation is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectrum spectrophotometer.

The antibodies of the present invention are particularly useful for the isolation of antigens by immunoprecipitation. Immunoprecipitation involves the separation of the target antigen component from a complex mixture, and is used to discriminate or isolate minute amounts of protein. For the isolation of membrane proteins cells must be solubilized into detergent micelles. Nonionic salts are preferred, since other agents such as bile salts, precipitate at acid pH or in the presence of bivalent cations.

In an alternative embodiment the antibodies of the present invention are useful for the close juxtaposition of two antigens. This is particularly useful for increasing the localized concentration of antigens, *e.g.*, enzyme-substrate pairs.

The compositions of the present invention will find great use in immunoblot or Western blot analysis. The anti-apo(a) related antibodies may be used as high-affinity primary reagents for the identification of proteins immobilized onto a solid support matrix, such as nitrocellulose, nylon or combinations thereof. In conjunction with immunoprecipitation, followed by gel electrophoresis, these may be used as a single step reagent for use in detecting antigens against which secondary reagents used in the detection of the antigen cause an adverse background. This is especially useful when the antigens studied are immunoglobulins (precluding the use of immunoglobulins binding bacterial cell wall components), the antigens studied cross-react with the detecting agent, or they migrate at the same relative molecular weight as a cross-reacting signal.

Immunologically-based detection methods for use in conjunction with Western blotting include enzymatically-, radiolabel-, or fluorescently-tagged secondary antibodies against the toxin moiety are considered to be of particular use in this regard.

G. ABBREVIATIONS

The abbreviations used herein are:

Lp(a), lipoprotein(a);
RLp(a), reassembled Lp(a);
apo(a), apolipoprotein(a);
Lp(a-), Lp(a) devoid of apo(a);
VLDL, very low density lipoprotein;
LDL, low density lipoprotein;
TG, triglycerides;
CE, cholesteryl esters;
FC, free cholesterol;
PL, phospholipids;
PAGE, polyacrylamide gel electrophoresis;
GGE, native gradient gel electrophoresis;
PFGE, pulsed-field gel electrophoresis;
EACA, ε-aminocaproic acid;
DTE, dithioerythritol;
PMSF, phenylmethylsulfonylfluoride;
BHT, β-hydroxytoluene;
KI, Kallikrein inactivator;
EDTA, ethylenediaminetetraaceticacid;
β-ME, β-mercaptoethanol;
LBS, lysine-binding site;
Lys^-, deficient lysine-binding;
Lys^+, positive lysine binding;

BD, binding domain;

ELISA, enzyme-linked immunosorbent assay.

* * * * * * * * * *

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

NOVEL METHODS FOR PRODUCING apo(a) FROM Lp(a)

A. EXPERIMENTAL PROCEDURES

1. Chemicals and Reagents

Materials were purchased from the following sources: Cyanogen bromide (CNBr)-Sepharose™ 4B, EACA, Tween™ 20, phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), L-lysine, L-proline, dithioerythritol (DTE), β-mercaptoethanol (β–ME), phosphate buffered saline packets (PBS) and cross-linked phosphorylase molecular weight standards for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) from Sigma Chemical Company, St. Louis, MO; porcine pancreatic elastase (EC1.4.21.36) Type V, human leukocyte elastase (EC 3.4.21.37), human fibrinogen and human plasma fibronectin from Sigma Chemical Co. (St. Louis, MO); Kallikrein inactivator (KI) from Calbiochem, San Diego, CA; molecular weight standards for native gradient gels from Pharmacia-LKB (Alameda,

CA); lambda DNA size standards and polyacrylamide from Bio-Rad (Richmond, CA); Immobilon-P® membranes from Millipore (Bedford, MA) and an enhanced chemiluminescent kit (ECL Western Blotting Detection kit) from Amersham (Arlington Heights, IL). All other chemicals were of reagent grade.

Antisera to purified preparations of Lp(a) and LDL were raised in the rabbit and affinity-purified antibodies to apo(a), Lp(a) and LDL were prepared as previously described (Fless *et al.*, 1989). Anti-Lp(a) were shown to be devoid of immunoreactivity to LDL and plasminogen, and anti-LDL were unreactive to Lp(a) and apo(a). Monoclonal antibodies to apo(a) KV were prepared in the inventors' laboratory.

2. Buffers

Buffer A was 10 mM phosphate containing 1 mM EDTA, 0.02% NaN_3, pH 7.5. Buffer B was 10 mM phosphate containing 1 mM EDTA, 0.02% NaN_3 and 100 mM EACA, pH 7.5. All other buffers were prepared as described in the text.

3. Human Subjects Used in the Study

The four wild-type subjects were two males (one Afro-American, one Caucasian) and two females (Caucasians), all healthy with Lp(a) protein levels in the range of 15-43 mg/dL (Table 2). All of them were heterozygous for apo(a) size isoforms based on protein and genomic analyses. One healthy subject (male, Caucasian) with the Trp72→Arg mutation in apo(a) kringle IV-10 had plasma Lp(a) protein levels of 0.16 mg/dL (Scanu *et al.*, 1994), (Table 2), and had a single allele and a single apo(a) isoform. The plasma from all subjects was obtained by plasmapheresis performed in the Blood Bank of the University of Chicago.

TABLE 2

Lp(a) Levels, Phenotypes and Genotypes of Wild-Type and Mutant Subjects

Subject	Lp(a) protein (mg/dL)[a]	Phenotype (kDa)[b]	Genotype (kb)[c]
K.B.	32	289; 488	58; 113
D.G.	15	379; 401	74; 103
B.K.	43	333; 341	69; 74
P.T.	35	330; 379	69; 73
T.T.	0.16	279	56

[a] Measured by ELISA as Lp(a) protein.

[b] The molecular weights of each of the two phenotypes from K.B. and one of the phenotypes from B.K. (341 kDa) were measured in the analytical ultracentrifuge (Fless *et al.*, 1994) and included the weight of the carbohydrates. The size of the phenotypes from D.G. and P.T. was estimated on Western blots of reduced SDS-PAGE.

[c] Allele size was estimated by PFGE in 1% agarose gels using the mobilities of lambda DNA standards in a 48.5 kb ladder encompassing a size range of 0.05 - 1 mb.

The steps for Lp(a) and LDL isolation were carried out immediately after blood drawing. The plasma samples used for the isolation of VLDL were obtained from either normolipidemic healthy human donors or dyslipidemic subjects (type IV) before receiving treatment at the University of Chicago Lipid Clinic and had plasma levels of Lp(a) protein below 1 mg/dL. The inventors used an additional 10 subjects for studying the apo(a) fragments in their plasma and urine. These were also healthy subjects with a known phenotype and genotype. Their plasma Lp(a) protein levels varied between 0.1 and 10 mg/dl and their size isoforms varied between 300 and 600

kDa. Aliquots of 3 ml of plasma were floated at d 1.21 g/ml in a TLA-100.3 rotor in a tabletop TL-100 ultracentrifuge (Beckman Instruments, Palo Alto, CA) for 18 h at 100,000 rpm. The top fraction containing Lp(a) and the bottom fraction containing apo(a) were quantitated by ELISA using monospecific apo(a) antibodies and also analyzed by Western blots of SDS-PAGE. In each subject, 3-4 h urine samples were collected, centrifuged for 15 min at 3000 rpm and either used fresh or frozen immediately at -80°C. Before use, they were thawed, concentrated 200 fold in microconcentrators (Amicon Corp. Beverly, MA) and then separated by SDS-PAGE.. . All of the subjects used in the study gave a written informed consent.

4. Phenotyping and Genotyping of Apo(a)

Apo(a) phenotyping was performed on either reduced plasma, isolated apo(a) or Lp(a) samples by SDS-PAGE followed by immunoblotting using anti-Lp(a) (Edelstein *et al.*, 1995). The mobility of the individual apo(a) bands was compared with isolated apo(a) isoforms of known molecular weights (Fless *et al.*, 1994). For apo(a) genotyping, DNA plugs were prepared from blood mononuclear cells, subsequently fractionated by pulsed field electrophoresis and the blots probed with an apo(a) specific probe essentially as described earlier (Lackner *et al.*, 1991).

5. Preparation of VLDL and LDL

For the isolation of VLDL, the plasma sample from wild-type and mutant subjects and from rhesus monkeys were adjusted with 0.01% NaN_3, 10,000 U/L of KI and 1 mM PMSF and centrifuged at d = 1.006 g/mL at 412,160 x *g* in a Beckman TLA100.3 rotor for 3 h at 15°C. The floating fraction was placed under an equal volume of saline and respun under the same conditions. The final floating VLDL was tested for the presence of apo(a) by Western blots of SDS-PAGE gels and stored at 4°C in saline containing 2 mM PMSF and the same inhibitors, under nitrogen, and utilized within 24 h. LDL were isolated at d = 1.030-1.050 g/mL by sequential flotation as previously described (Schumaker and Puppione, 1986).

6. Preparation of Human Wild-Type and Mutant Lp(a)

To prevent lipoprotein degradation, the plasma obtained by plasmapheresis was adjusted with 0.15% EDTA, 0.01% NaN_3, 10,000 U/L KI and 1 mM PMSF. Wild-type Lp(a) were isolated by sequential ultracentrifugation and lysine-Sepharose™ chromatography as previously described (Fless *et al.*, 1994). For the isolation of mutant Lp(a), the plasma was brought to d = 1.050 g/mL with solid NaBr, and proline at a final concentration of 100 mM was added to prevent the interaction of LDL with the small amounts of Lp(a) present in these subjects. The solution was ultracentrifuged at 302,120 x *g* for 20 h at 15°C, the floating fraction was removed and the density of the bottom fraction adjusted to d = 1.10 g/mL and recentrifuged under the same conditions. Subsequently, the floating fraction was dialyzed against 10 mM Tris-HCL, pH 7.5 and subjected to FPLC ion-exchange chromatography as described previously (Scanu *et al.*, 1993). The purity of the product was assessed by mobility on precast 1% agarose gels (Ciba-Corning, Palo Alto, CA) and Western blots of SDS-PAGE, utilizing anti-Lp(a) and anti-apoB. The purified Lp(a) were filter sterilized and stored at 4°C. An aliquot of both blood and plasma from each subject was utilized for apo(a) genotyping and phenotyping, respectively. In three of the subjects, D.G. B.K. and P.T. the two apo(a) phenotypes were close in size (Table 2); thus Lp(a) species containing a single

phenotype could not be separated by the procedure used. On the other hand, in the case of K.B., the high differential in apo(a) size and thus Lp(a) density permitted the separation of two Lp(a)s, one containing the 289 kDa and the other the 488 kDa phenotype. The technique used was an adaptation of that described by Fless *et al.*, (1994).

7. Preparation of Rhesus Monkey Lp(a)

The four rhesus monkeys studied were from the same pedigree previously described (Scanu *et al.*, 1993). All had high plasma Lp(a) protein levels varying between 20 and 40 mg/dL, exhibited a single band phenotype and were housed at the Southwest Foundation for Biomedical Research in San Antonio, TX. The monkeys were fasted overnight before collecting 20 mL of venous blood in tubes containing 0.01% EDTA. The isolation procedure was essentially as described previously (Scanu *et al.*, 1993). Since rhesus apo(a) is Lys^-, the inventors could not utilize lysine-Sepharose™ chromatography for the isolation of Lp(a). In brief, the plasma was spun at 302,120 x *g* at 10°C for 20 h at d = 1.050 g/mL. After removal of the top layer, the infranatant was adjusted to d = 1.070 g/mL with solid NaBr and spun under the same conditions. The top layer containing mainly Lp(a) was removed, dialyzed against 10 mM Tris-HCL, 0.01% EDTA, 0.01% NaN_3 and subjected to FPLC ion-exchange chromatography as fully described by Scanu *et al.*, (1993). The isolated Lp(a) were filter sterilized and stored refrigerated in 33 mM phosphate buffer containing 2 mM PMSF, 0.15% EDTA and 0.01% NaN_3, pH 7.5 under nitrogen.

8. Dissociation and Isolation of Apo(a) from Lp(a)

Lp(a), 1 mg/mL protein (2 mL), was incubated with DTE at a final concentration of 1.5-2 mM in Buffer A for 10 min under argon gas at room temperature. EACA to a final concentration of 100 mM was then added in small increments and the reaction mixture was protected from light with aluminum foil and rotated slowly (7 rpm) in a general purpose rotator at room temperature for 1 h under nitrogen gas.

Subsequently, the incubated mixture was dialyzed for 2 h at room temperature against 2 changes of 4 liters each of Buffer B purged with nitrogen gas. After dialysis, an equal volume of 60% sucrose in Buffer B was added and the resulting mixture distributed into Beckman polycarbonate tubes, so that about 0.5 mg of free apo(a) was contained in each tube, placed into a TLA 100.3 titanium rotor and spun in a table top TL100 ultracentrifuge at 10°C, 412,160 x *g* for 18 h at acceleration and deceleration settings of 6. After centrifugation, the top 0.5 mL fraction contained LDL free of apo(a), (Lp(a-)) and unreacted Lp(a) and the bottom 1.0 mL fraction contained free apo(a) in pure form. The latter was stored in the sucrose solution at -80°C.

The yield of free apo(a) was 90-100%. When 0.5 and 1 mM DTE was used, while maintaining the other experimental conditions unchanged, the resulting apo(a) behaved in a similar way but the yields, based on the ELISA of the Lp(a) remaining in the floating fraction, were significantly lower than those obtained with 2 mM DTE; about 55 and 70% with 0.5 and 1 mM DTE, respectively *vs.* 90% with 2 mM DTE. The disassembly process was the same whether starting from Lp(a) preparations containing either one or two phenotypes.

9. Reassembly of Lp(a) from Apo(a) and LDL

Apo(a), (1 μg) of a defined phenotype obtained from human Lp(a) by the procedure outlined above, was incubated with an homologous preparation of LDL in buffer A at different apoB100:apo(a) molar ratios in a total volume of 175 μL (final apo(a) concentration was 5.7 μg/mL) in a shaking water bath at 37°C for various time intervals in the presence of 50 μM BHT, KI (10,000 KIU/mL) and 1 mM PMSF, under nitrogen. Aliquots of the reaction mixture were then analyzed on Western blots of SDS-PAGE gels under non-reducing and reducing (3% β-ME for 5 min at 95°C) conditions. In some studies the apo(a) was incubated with 100 mM EACA for 60 min at 37°C before the addition of LDL and the mixture then incubated for the desired time. To quantitate the amount of Lp(a) assembled, an aliquot (125 μL) of the reaction mixture was diluted with an equal volume of 60% sucrose in buffer A containing 200 mM

EACA and spun in a TLA100 rotor (tube capacity, 250 μL) at 412,160 x *g* at 15°C for 18 h. The top fraction (105 μL) was removed and quantitated by ELISA designed to measure the apoB100:apo(a) complex (Fless *et al.*, 1989). The bottom 100 μl containing free apo(a) was also quantitated by a sandwich ELISA specific for apo(a) using anti-Lp(a) for coating and alkaline phosphatase-conjugated anti-Lp(a) for detection.

In the studies directed at defining the properties of RLp(a), 1-2 mg of apo(a) was incubated with LDL in Buffer A containing 2 mM PMSF at an apoB100:apo(a) molar ratio of 50:1 at 37°C for 24 h under nitrogen. The reaction mixture was centrifuged overnight in 30% sucrose in Buffer B, d = 1.127 g/mL, the floating fraction was dialyzed against Buffer A and RLp(a) isolated from unreacted LDL by lysine-Sepharose™ chromatography, or, in the case of the human mutant and rhesus monkeys by FPLC. Alternatively, the reassembly reaction mixture, after dialysis against Buffer B, was subjected to density gradient ultracentrifugation (Nilsson *et al.*, 1981) which effects an efficient separation of LDL from Lp(a) and apo(a). The fractions containing Lp(a) were dialyzed against Buffer A and further purified to homogeneity by affinity chromatography with lysine-Sepharose™ or FPLC.

10. Reassembly of Lp(a) from Apo(a) and VLDL

The procedure was the same as that described for the reassembly with LDL. The molar ratio of apoB100 to apo(a) was based on the concentration of apoB100 in VLDL as measured by ELISA.

11. Preparation of Fully Reduced and Alkylated Apo(a)

To reduce all the disulfide bonds, apo(a) was incubated in Buffer A containing 50 mM DTE, 6 M guanidine HCl for 2 hrs at room temperature. The mixture was made 150 mM with respect to iodoacetamide and the incubation continued for an additional hour. Before use, the reduced and alkylated apo(a) was dialyzed against buffer A.

12. Limited proteolysis of Lp(a) and apo(a)

To optimize the conditions for limited proteolysis, the inventors conducted studies in which Lp(a) and apo(a), containing KI (200 U/ml), were incubated at 20°C with porcine pancreatic elastase at various molar ratios of Lp(a) or apo(a) protein to enzyme (5, 10, 25, 50, 100 to 1) for time periods of 1 to 24 h. The digestion mixtures were then examined on Western blots of SDS-PAGE probed with anti-apo(a). The inventors' end point for limited proteolysis was the production f two major bands of 220 kDa and 170 kDa. Minor amounts (<10%) of smaller molecular weight bands were also observed. The final conditions used in the subsequent studies were as follows: Lp(a) or apo(a) in 50 mM Tris-HCI, 100 mM NaCl, pH 8.0, LI (200 U/ml) were digested with pancreatic elastase at a molar ratio of 25:1 (protein:enzyme) at 22°C for 2 h and the reaction terminated by the addition of 5 mM DFP with further incubation for 20 min. Limited proteolysis with human leukocyte elastase (1 U = 1nm p-nitrophenol/sec from N-1BOC-L-Ala p-nitrophenyl ester) was conducted after 1500 fold dilution of the enzyme in Tris buffer without KI. One microliter of the diluted enzyme was incubated per μg of apo(a) protein at 37°C for one hour. The reaction was terminated with 5 mM DFP for 20 min at 22°C.

13. Lysine-Sepharose™ Chromatography

CNBr-activated Sepharose™ 4B was coupled to the α-amino group of lysine essentially according to the instructions supplied by Pharmacia-LKB. The amount of lysine crosslinked to the beads was assessed as described (Wilkie and Landry, 1988) and ranged between 16 and 21 μmoles of lysine per mL bead suspension. Chromatography was performed at room temperature on a Bio-Rad Econo® Chromatography system. Columns were packed with lysine-Sepharose™ at a ratio of 5 mL of packing material to 1 mg of Lp(a) protein and equilibrated with PBS containing 1 mM EDTA, and 0.02% NaN_3. After loading, the column was washed with at least 3 column volumes of

equilibrating buffer at the same flow rate. Fractions containing apo(a) or Lp(a) were pooled and dialyzed against Buffer A.

14. Molecular Sieving Chromatography

To separate undigested apo(a) from the elastase digest, 0.2 ml aliquots were applied onto a Superose 6 prepacked HR 10/30 column (Pharmacia Biotech Inc., Piscataway, NJ.) previously equilibrated with 10 mM Tris-HCl, pH 8.0 containing 150 mM NaCl. Chromatography was conducted with an FPLC system (Pharmacia Biotech) at a flow rate of 0.3 ml/min.

15. Fibrinogen and fibronectin binding assay

The wells of microtiter plates (Beckman, Fullerton, CA) were coated with 10 µg/ml (100 µl/well) of fibrinogen or fibronectin in TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated overnight at 22°C. Nonspecific binding sites were blocked with 2% BSA in TBS for 2 h at 22°C. After washing with TBST buffer (TBS supplemented with 0.1% BSA and 0.02% Tween-20), various concentrations of Lp(a) and the derived fragments were added to the wells in TBS buffer with or without 200 mM EACA and incubated for 2 h at 22°C. After washing with TBST buffer, rabbit anti-apo(a) antibody was added and incubated for 1 h at 22°C. At this time, the wells were washed with TBST buffer and the goat anti-rabbit IgG conjugated to alkaline phosphatase was added for 1 h at 22°C. After washing with TBST buffer, p-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer, pH 9.8) was added and the color development followed at 405 nm on a microplate reader, Biomek 100 (Beckman, Fullerton, CA). The results obtained as the change of absorbence per minute, were then transformed into moles by using standard curves established for each component. The maximum number of moles bound at saturation was defined as B_{max}. Analysis of the binding data was performed on the assumption of single-site binding. Dissociation constants (Kd) were derived from the slope of the linearized expression of the Langmuir equation (Fleury and Angles-Cano, 1991).

$$[Fn \bullet X]=[Fn_o]\frac{K[X[}{(1+K[X])}$$

where $[Fn_o]$ represents the total number of fibrinogen or fibronectin binding sites, [Fn] the number of moles of Lp(a) or apo(a) adsorbed on fibrinogen or fibronectin, [X] refers to Lp(a), apo(a), mini-Lp(a) or apo(a) fragments and K the association constant.

16. Electrophoretic Methods

SDS-PAGE, (3.5% separating gel, 2.75% stacking gel), was performed on a Novex system (Novex, San Diego, CA) for 1.5 hr at constant voltage (120 V) at room temperature. The samples were prepared by heating at 95°C for 5 min in sample buffer which consisted of 94 mM Phosphate buffer, pH 7.0, 1% SDS and 2 M urea with or without 3% β-ME. Immediately after electrophoresis, the gels were placed onto Immobilon-P® sheets which were previously wetted with a buffer containing 48 mM Tris, 39 mM glycine, pH 8.9. Blotting was performed on a horizontal semi-dry electroblot apparatus (Pharmacia-LKB, NJ) at 0.8-1 mA/cm^2 for 45 min at room temperature. To assess the size and integrity of LDL, Lp(a), and RLp(a), non-denaturing PAGE (GGE) was performed on precast 2.5-16% polyacrylamide slab gels (Isolab, Akron, OH) as described (Nichols *et al.*, 1986).

17. Immunoblotting

After electroblotting, the Immobilon-P® blots were blocked in PBS containing 5% non-fat dry powdered milk and 0.3% Tween® 20 followed by incubation with anti-Lp(a) or anti-apoB antibody. In specified cases, monoclonal antibodies directed against kringle V of apo(a) were used. The blots were washed and incubated with anti-rabbit horseradish peroxidase-labeled IgG. Subsequently, the blots were developed with the ECL Western Detection Reagent according to the manufacturer's instructions.

18. Amino Acid Analyses

Amino acid analyses were performed at the University of Kentucky Macromolecular Structure Analysis Facility. The mildly (2 mM DTE) and fully reduced and alkylated apo(a) were dialyzed against a 10 mM solution of 4-ethylmorpholine acetate, pH 8.0, and lyophilized for shipment. The moles of free sulfhydyls in apo(a) that were alkylated was determined as carboxymethylcysteine.

(a) Amino-terminal sequence analyses

Apo(a) fragments (10-30 μg) were electrophoresed under reducing conditions as outlined above. After electrophoresis, the gels were electroblotted onto Immobilon PSQ sequence grade membranes (Millipore Corp., Bedford, MA) as described above in the immunoblotting section. The blots were rinsed in distilled water, stained with Coomassie Blue R250 (0.025% in 40% methanol) and destained with 50% methanol. The stained bands were cut from the membrane, further washed with 40% methanol and allowed to air dry. Reduction with DTT and alkylation with iodoacetamide was performed directly on the PSQ membrane which was then subjected to automated Edman degradation on an Applied Biosystems 477A unit using procedures recommended by the manufacturer.

19. Electron Microscopy

Solutions containing lipoproteins at 0.05 mg/mL protein in 10 mM NH_4HCO_3 were transferred to Formvar-carbon coated copper grids. Lipoproteins were allowed to adhere, and the excess was removed by touching the edge of the grids with filter paper. After washing the grids twice with deionized water, each grid was coated with one drop of 1% phosphotungstic acid. The excess phosphotungstic acid was removed and the grids were air dried and examined in a Philips CM 10 electron microscope at an accelerating voltage of 100 kV.

20. Circular Dichroic Measurements

CD spectra were measured on a Jasco J-600 spectropolarimeter (Jasco, Japan) and analyzed using the J-700 software after conversion of the data using the Softsec File conversion program (Softwood Co., CT). Spectra were recorded at protein concentrations ranging from about 0.1 mg/mL to 2 mg/mL in cuvettes of 0.01 to 0.1 cm path length. Mean residue ellipticities were calculated using the following mean residue weights: 112.8 for apo(a) and 113.3 for Lp(a) and RLp(a). The secondary structure content was calculated by two methods, using the program VARSLC1 starting with a set of 33 reference proteins (Manavalan and Johnson, 1987), and the program CONTIN (Provencher and Glockner, 1981). All samples were previously dialyzed against Buffer A but without PMSF since this reagent interfered with CD absorption in the far ultraviolet region of the spectrum.

21. Lipid, Lp(a) and apo(a) Analyses

Total cholesterol and free cholesterol were measured with enzymatic kits from Boehringer Mannheim (Indianapolis, IN). The mass of cholesteryl esters was the difference between total and free cholesterol multiplied by the factor 1.68. Triglycerides were determined by a test kit from Sigma (TG INT #336) and phospholipids as inorganic phosphorous using the Fiske and Subbarow reagent (Fisher, USA) following the method of Bartlett, (1959) and using the factor 25 to convert inorganic phosphate to phospholipid mass. Lp(a) and LDL protein were quantitated by a sandwich ELISA essentially as previously described (Fless *et al.*, 1989) except that anti-Lp(a) IgG was used as the capture antibody and anti-apoB IgG conjugated to alkaline phosphatase as the detection antibody. For the ELISA quantitation of apo(a), anti-apo(a) IgG conjugated to alkaline phosphatase was used 22 as the detection antibody. . Subsequently, an extinction coefficient (ε_{278} = 1.31 mL mg^{-1} cm^{-1}) was established for apo(a) in the 30% sucrose solution. Quantitation of kringle V in apo(a) and the fragments was carried out with a sandwich ELISA in which anti-kringle V was the capture antibody and anti-apo(a) IgG conjugated to alkaline phosphatase was the

detection antibody. Protein determinations were performed by the Bio-Rad DC Protein assay.

22. Metabolic studies in mice

Balb/c female mice (10-12 weeks) from Jackson Laboratories (Bar Harbor, ME) were used. All mice were housed in individual cages under normal light. The evening before the study, the mice were given a 10% sucrose solution to drink ad libitum in place of water. The following morning the mice were anesthetized with Metafane and 25-250 μg of either Lp(a), apo(a), or the fragments obtained from the elastase digestion in a volume of 200 μL were injected into the tail vein. The mice were then placed in metabolic cages, given access to standard lab chow and the 10% sucrose solution. Blood samples were withdrawn from the orbital vein into heparinized hematocrit tubes at the specified time points and immediately iced. Urine was collected at 0-3, 3-5 and 5-24 h. ELISA quantitation, sensitive to < 0.0015 mg/dl of apo(a), was performed on the urine samples to determine the levels of apo(a) reacting material. These results indicated to what degree the sample was to be concentrated for electrophoretic detection which was estimated to be > 0.03 mg/dl. The urine was concentrated in Amicon Centriprep filters and the extent of concentration for each representative sample is stated in the legends to the Figures. Mouse plasma and urine were analyzed for the levels of apo(a), Lp(a) and kringle V by a sandwich ELISA using monospecific antibodies as described above. The decay of Lp(a), apo(a) or fragments in plasma was expressed as:

$$\text{percent of injected dose} = (\text{mg/dl})_t / (\text{mg/dl})_i \times 100$$

where t was the concentration at a given time and i was the concentration in plasma at 1 min after injection. The log of the percent of the injected dose was plotted against time and the half-time ($T_{1/2}$) of the injected sample in the intravascular compartment was determined from the slope of the linear portion of this curve. The plasma and urine specimens were also analyzed by SDS-PAGE.

23. Molecular modeling

Modeling was performed on a molecular graphics workstation from Silicon Graphics Inc. using the modeling system Insight II v.95.0 and the programs Buider, Biopolymer and Discover (Biosym/MSI, San Diego, CA). Since crystallographic coordinates are not available for the linker regions the inventors used the amino acid sequence deduced from the cDNA sequence (McLean *et al.*, 1987) and built each amino acid sequentially in a linear fashion. The secondary structure was then constructed based on the algorithms of Chou-Fasman (1978) and Gamier/Robeson (1978) and the model was subjected to energy minimization.

B. RESULTS

1. Studies with Human Wild-Type Lp(a) Properties of Isolated Apo(a)

The Lp(a) from subjects containing two apo(a) phenotypes (488 and 289 kDa) was separated into two Lp(a) species each containing one of the two phenotypes and then incubated with 2 mM DTE in the presence of 100 mM EACA. The resulting products were isolated at d = 1.127 g/mL in 30% sucrose and analyzed on Immobilon-P blots of 3.5% polyacrylamide SDS-PAGE gels followed by immunoblotting with mono-specific anti-Lp(a) or anti-apoB100 antibodies and visualized by chemiluminescence. The Western blots of the products dissociated from Lp(a) by the action of DTE showed that only free apo(a) was present in the sedimenting fraction but not in the floating fraction which contained only apoB100 and barely detectable quantities of unreacted Lp(a). Reduction with 3% β-ME caused the apo(a) bands of the two phenotypes, 488 and 289 kDa, to shift to a position in the gel corresponding to that of the parent reduced Lp(a). In terms of isoform number, the pattern of the isolated free apo(a) resembled that of the parent Lp(a).

The isolated apo(a) could be stored for at least 2 months at -80°C in a 30% sucrose solution containing 100 mM EACA, without apparent changes in properties based on CD analyses and lysine binding (see below). However, lysine binding decreased significantly (up to 40% in two weeks) when the isolated apo(a) was stored only in buffer A at -80°C. Based on amino acid analyses, apo(a) contained 3 ± 2 (n=7) moles of cysteine per mole of protein (289 kDa isoform) as compared to 100 ± 7 (n=7) for the fully reduced and alkylated product. The theoretical number of fully reduced cysteines was calculated to be 106. The isolated apo(a) examined by far-ultraviolet CD spectroscopy gave a spectrum (FIG. 1) characterized by a strong negative band at 203 nm and a positive band at 222-232 nm. Although the CD deconvolution programs are mainly suited to globular proteins, these values agreed with those predicted for apo(a) by Guevara *et al.*, (1992). Free apo(a) bound to a lysine-Sepharose™ column and could be eluted from it only with 200 mM EACA but not with PBS (FIG. 2C), a behavior similar to that of the parent Lp(a) (FIG. 2A).

2. Reassembly Studies

(a) Reassembly of Lp(a) from Free Apo(a) and LDL

In order to limit the number of variables the inventors used LDL preparations which were homogeneous on non-denaturing GGE and banded in the density range of 1.030-1.050 g/mL. The apo(a) size isoforms studied (Table 2) gave comparable results in terms of forming RLp(a). The yields were comparable among all the apo(a) isoforms except for the high molecular weight 488 kDa phenotype from subject K.B. which, on a molar basis, associated comparatively less efficiently with LDL (see below).

Apo(a) from subject D.G. was incubated with LDL (subject D.G.) at an apoB100:apo(a) molar ratio of 25:1 for 24 h at 37°C and the reassembled Lp(a) separated by lysine-Sepharose™ chromatography and analyzed on Western blots of 3.5% polyacrylamide slab SDS-PAGE gels with antibodies to Lp(a) and apoB100. Lanes 1-3, anti-Lp(a) blots of unreduced gels of parent Lp(a), free apo(a) and RLp(a)

respectively; lanes 4-6, anti-apoB100 blots of unreduced gels of parent Lp(a), RLp(a) and control LDL respectively; lanes 7-9, blots of reduced samples as in lanes 1-3; lanes 10-12, blots of reduced samples as in lanes 4-6. Markers for Lp(a) and apo(a) are shown on the left of the unreduced gels and apo(a) and apoB100 on the right of the reduced gels.

Western blots probed with anti-apo(a) and anti-apoB100, showed that the band corresponding to RLp(a) contained both protein components even though the sample had been boiled in SDS prior to gel electrophoresis, suggesting a covalent association between apoB100 and apo(a). This was corroborated by the finding that samples reduced with 3% β-ME, generated two bands corresponding to free apo(a) and apoB100. The percent of apo(a) which was recovered in the RLp(a) was a function of both time of incubation and the initial apoB100:apo(a) molar ratios in the mixture. At a molar ratio of 50:1, 50% of the initial apo(a) mass became RLp(a) in the first hour (FIG. 3A). The progressive increase in the reassembly process reached a plateau after 5 h at which time 75% of apo(a) was associated with apoB100. At a molar ratio of 25:1 it took a longer time to reach similar levels of reassembly. Varying the initial apo(a) concentration (5.7, 11.4, 28.6 and 45.9 μg/mL) in a system with a 50:1 apoB100:apo(a) molar stoichiometry had no significant effect on Lp(a) reassembly. In terms of size polymorphism, the high molecular weight isoform (488 kDa) gave an Lp(a) reassembly efficiency which was two-fold lower than that with the 289 kDa isoform.

Lysine and proline have been previously reported to inhibit the assembly of Lp(a) (Chiesa *et al.*, 1992; Phillips *et al.*, 1993; Trieu *et al.*, 1991). In this system, incubation of apo(a) with either of the two reagents prior to incubation with LDL significantly inhibited Lp(a) reassembly. In each case, at 500 mM, a maximum inhibition of 90% was attained. At concentrations equal to or below 200 mM, EACA appeared to be a relatively more potent inhibitor than proline (Table 3). On the premise that EACA and proline interact at different sites on apo(a) (Trieu *et al.*, 1991), apo(a) with 100 mM each of EACA and proline prior to incubation with LDL. Under these conditions a two-fold higher inhibition of the Lp(a) reassembly, was obtained as

compared to the incubation with a single inhibitor (Table 3). Moreover, when apo(a) was fully reduced with 50 mM DTE, dialyzed and incubated with LDL, less than 5% of the total apo(a) was reassembled. In addition, when the fully reduced apo(a) was alkylated and applied to a lysine-Sepharose™ column, all of the apo(a) was recovered in the unbound flow through fraction.

TABLE 3

Effect of EACA and Proline on the *in vitro* Reassembly of Lp(a) from Human Wild-Type and Rhesus Monkey Apo(a) and Human LDL[a]

	(% ± SD)
Human apo(a) + LDL	100[b]
Human apo(a) + LDL + EACA	
pre -incubation[c]	18.8 ± 5.4
post -incubation[d]	88.5 ± 15.8
Human apo(a) + LDL + proline	
pre -incubation	28.7 ± 6.3
post -incubation	93.5 ± 10.3
Human apo(a) + LDL + proline + EACA[e]	10.0 ± 2.0
Rhesus apo(a) + LDL	100[b]
Rhesus apo(a) + LDL + EACA	
pre -incubation[c]	15.0 ± 5.3
post -incubation[d]	86.2 ± 16.2
Rhesus apo(a) + LDL + proline	
pre -incubation	30.3 ± 7.1
post -incubation	94.7 ± 9.3
Rhesus apo(a) + LDL + proline + EACA[e]	10.2 ± 3.1

[a] All incubations were performed using human or rhesus apo(a) and human LDL at an apoB100:apo(a) molar ratio of 50:1, for 5 h at 37°C. After centrifugation overnight in sucrose at d 1.127 g/mL, the floating fraction was analyzed for the presence of apoB100:apo(a) complexes by ELISA as described.

[b] For ease of comparison, the fraction of the total apo(a) covalently linked to apoB100 was given a value of 100. All of the percentages were calculated relative to this value and were the results of 5 studies.

[c] EACA or proline (100 mM) was preincubated with apo(a) for 1 h at 37°C before incubation with LDL.

[d] EACA or proline (100 mM) was added after the complex was formed and incubated for 1 h.

[e] Both EACA and proline, each at 100 mM, were preincubated with apo(a) for 1 h at 37°C before interaction with LDL.

(b) Properties of Lp(a) Reassembled from Apo(a) and LDL

When apo(a) was incubated with LDL at an apoB100:apo(a) molar ratio of 25:1 for 6 h, and the mixture fractionated by isopycnic density gradient ultracentrifugation RLp(a) banded in the same density position as the parent Lp(a). Regardless of the procedure of isolation, RLp(a) bound to lysine-Sepharose™ and was eluted from it with 200 mM EACA in a manner comparable to native Lp(a). By electron microscopy (FIG. 4A, FIG. 4B and FIG. 4C), the RLp(a) particles were slightly heterogeneous with an average diameter of 27.9 nm ± 4.0 S.D. (n=115) very similar to their native Lp(a) counterparts (average diameter, 28.4 nm ± 4.1 S.D. (n=115). The LDL utilized for the reassembly showed a similar heterogeneity with an average diameter of 27.5 nm ± 3.5 S.D. (n=130). On GGE, RLp(a) had the same mobility as control Lp(a) indicating that both had a similar size or Stokes' radius. Moreover, as measured by circular dichroism, the conformation of RLp(a) closely resembled that of native Lp(a) (FIG. 1). The spectra of both lipoproteins were characterized by negative bands at 218 nm and 210 nm. Secondary structure calculations performed using the programs VARSLC1 and CONTIN gave 24% α-helix, 29% β-sheet and 47% random structure for RLp(a), and 23% α-helix, 31% β-sheet and 46% random structure for Lp(a). The chemical composition of the RLp(a) was also comparable to that of the parent Lp(a) and had the same lipid composition as the LDL preparation used in the reassembly system (Table 4).

Based on ELISA analysis of apoB100 and the apo(a):apoB100 complex in RLp(a), the molar stoichiometry of apoB100:apo(a) was calculated to be 1:1.

TABLE 4

Chemical Composition of RLp(a) and Control Lipoproteins[a]

	% ± SD				
	PL	CE	FC	TG	RLp(a)
Protein	26.0 ± 1.5	17.2 ± 2.0 (23.2)[b]	41.6 ± 2.3 (56.2)	8.8 ± 1.4 (12.0)	6.4 ± 1.3 (8.6)
Lp(a)	26.9 ± 1.7	14.6 ± 1.6 (20.1)	42.9 ± 2.0 (58.6)	11.0 ± 1.2 (15.0)	4.6 ± 0.8 (6.3)
LDL	23.0 ± 1.3	17.6 ± 1.7 (22.9)	44.5 ± 1.8 (57.7)	10.3 ± 1.1 (13.3)	4.6 ± 0.5 (6.1)

[a] Lp(a) was isolated from plasma and utilized for the preparation of apo(a). The latter and LDL were used to prepare RLp(a). Apo(a), Lp(a) and LDL were obtained from subject K.B. with the 289 kDa phenotype. The percent was calculated from four studies;

[b] Values in parentheses correspond to the lipid distribution only.

The effect of EACA and proline on the stability of RLp(a) was also assessed. After RLp(a) was formed, it was incubated with 100 mM of either EACA or proline for 1 h at room temperature. The mixture was then centrifuged at d = 1.127 g/mL for 18 h and the floating fraction containing the apoB100:apo(a) quantified by ELISA. Addition of either EACA or proline caused some apo(a) to dissociate [10-12% of the total RLp(a)] (see Table 3), indicating that a small portion of apo(a) complexed with apoB100 through non-covalent interactions and co-purified with the stable disulfide-linked complex. On the other hand, when 2 mM DTE was added to RLp(a) there was an almost complete dissociation of apo(a) from apoB100.

3. Reassembly of Lp(a) from Apo(a) and VLDL

It has been shown that apo(a) linked to apoB100 can be found in triglyceride-rich particles isolated from hyperlipidemic plasma (Scanu *et al.*, 1992). The *in vitro* interaction of apo(a) isolated from an Lp(a) having a single apo(a) isoform (289 kDa) was examined with preparations of VLDL isolated from the plasma of two hypertriglyceridemic subjects (R.W. and R.Z.) with type IV dyslipoproteinemia and very low plasma levels of Lp(a) protein, *i.e.*, 0.1 and 0.3 mg/dL, respectively. The experimental conditions for the reassembly were as described for the LDL studies using a 50:1 apoB100:apo(a) molar ratio. In the early phase (up to 2.5 h) the reassembly process between apo(a) and VLDL followed a course (FIG. 3B) which was similar to that observed between apo(a) and LDL (FIG. 3A). However, VLDL required 13 h to reach the 75% reassembly level which in the case of LDL was achieved in 5 h. As in the case of LDL, the reassembly of apo(a) with VLDL was inhibited by 100 mM EACA or proline (14% and 23% of apo(a) reassembled, respectively, compare with data in Table 3). Moreover, like the reassembled product from apo(a) and LDL, the one obtained from the interaction between apo(a) and VLDL was only dissociable by 2 mM DTE, indicating that a disulfide linkage had been formed.

4. Properties of Human Mutant Apo(a) and RLp(a)

In spite of the fact that subject T.T. had very low plasma levels of Lp(a), (0.16 mg/dL, Table 2), it was possible to obtain by plasmapheresis sufficient amounts of plasma to isolate Lp(a) and prepare apo(a) in a 200 μg yield. As with the human wild-type, the mutant free apo(a) was water-soluble and stable when stored in 30% sucrose and 100 mM EACA at -80°C. In terms of binding to lysine-Sepharose™, parent Lp(a) was Lys$^-$, (Scanu *et al.*, 1993), and thus eluted in the flow through volume. In contrast, free apo(a) bound effectively to the column and was eluted specifically with EACA similar to Rhesus apo(a), (see below). Incubation of apo(a) with either autologous or wild-type LDL at an apoB100:apo(a) molar ratio of 50:1 for 5 h at 37°C caused 60% of

the initial apo(a) to become complexed to LDL, as assessed by ELISA. Western blot analyses of the fraction floating in sucrose at d = 1.127 g/mL showed that the apoB100:apo(a) complex was sensitive to reduction by β-ME. As with the wild-type, both EACA and proline prevented reassembly between mutant apo(a) and LDL.

5. Properties of Rhesus Apo(a) and RLp(a)

Rhesus Lp(a), after reduction with 2 mM DTE yielded an apo(a) which was water-soluble and exhibited properties which were very similar to the human mutant apo(a). By chromatography on lysine-Sepharose™, rhesus Lp(a), due to the Trp72-Arg substitution in apo(a) kringle IV-10, was incompetent to bind and was eluted in the flow through PBS buffer (FIG. 2B). On the other hand, apo(a), once freed of its LDL moiety, bound tightly to the column and could only be eluted with EACA (FIG. 2C). Rhesus apo(a), at an apoB100:apo(a) molar ratio of 50:1, also bound equally to either autologous LDL or rhesus Lp(a-) or human wild-type LDL. The reaction between rhesus apo(a) and human LDL followed a time course of reassembly (FIG. 3A) similar to that of the wild-type human apo(a), reaching a maximum of about 60% in 5 h. Western blot analyses of the reassembled fraction which was contained in the d = 1.127 g/mL floating fraction, showed that an apoB100:apo(a) complex was formed and only dissociated in the presence of β-ME. EACA and proline inhibited the reassembly, although inhibition by either of these reagents was slightly more efficient than in the human model (Table 3).

C. DISCUSSION

A novel method for isolating wild-type and mutant apo(a) phenotypes in a free form has been developed by subjecting each parent Lp(a) to mild reductive conditions using 2 mM DTE and 100 mM of the lysine analogue, EACA. This procedure caused the cleavage of the interchain disulfide between one of the unpaired cysteines (Cys-3734,4190,4300) of apoB100 and the unpaired Cys-4057 in apo(a) kringle IV-9 without an apparent disruption of the intrachain disulfides in the kringles of apo(a). By amino

acid analyses, the apo(a) freed from Lp(a) had three cysteine residues instead of the single one which would have been expected if the mild reductive conditions had acted only on the interchain disulfide between apo(a) and apoB100. The "extra" two cysteines, rather than from kringles, might have derived from one of the 6 disulfides located in the protease region of apo(a), on the assumption that this region represents a comparatively more open structure than that comprising the kringles (Scanu and Edelstein, 1995) and, thus, potentially more amenable to disulfide cleavage.

The isolation of a water-soluble, functionally competent apo(a) is an important development particularly when one considers that essentially all apo(a) in the plasma is covalently linked to apoB100 and that much of the current information on apo(a) has been derived from results on recombinant products which may not necessarily reflect the properties of "native" apo(a). This development was dependent on various factors: 1) the use of very low concentrations of DTE; 2) the presence in the reaction mixture of EACA for the purpose of inhibiting the reassociation between apo(a) and LDL, 3) at the end of the reaction, the use of sucrose in order to achieve the necessary medium density for separating apo(a) from the LDL moiety by ultracentrifugal flotation. Sucrose had to be used because in its absence, apo(a) came out of solution at the NaCl concentrations required to float LDL and any unreacted Lp(a). Moreover, sucrose proved to be a good stabilizing factor in storing free apo(a) at -80°C. The ability to prepare relatively large amounts of free apo(a) of a defined phenotype, provides a powerful tool for gaining a broader knowledge on the structural properties of this unique glycoprotein and for defining, on a physiological level, its role in the process of Lp(a) assembly.

An important finding was that regardless of the functional state of the LBS of kringle IV-10 (*i.e.*, Lys^+ and Lys^-) of the parent Lp(a), all free apo(a)s bound specifically to lysine-Sepharose™ in that they could only be eluted by the lysine analogue, EACA. This indicates that the detachment from apoB100, opened in apo(a) a second domain which contained sites for both lysine and proline normally buried in native Lp(a). These sites may be either independent from each other or, may act in a cooperative way *via* the interkringle linkers. This is further corroborated by the finding that preincubation of the

free apo(a)-LDL mixture in the presence of equimolar concentrations (100 mM each) of EACA and proline resulted in a two-fold decrease in Lp(a) reassembly compared to the studies in which each inhibitor was used alone.

The effect of proline on the interaction between apo(a) and apoB100 was first recognized by Trieu *et al.*, (1991) and in a baboon cell system by White and Lanford, (1994). From the modeling studies of Guevara *et al.*, (1993), kringle IV-4 and/or kringle IV-9 by containing a hydrophobic pocket may be able to accommodate bulky hydrophobic amino acid residues such as proline. On the other hand, Ernst *et al.*, (1995), by studying human apo(a) mutant constructs expressed in HepG2 cells, identified a site spanning from kringle IV-5 through IV-9 which were believed to be involved in interaction between apo(a) and apoB100. The assignment of this site, however, must be viewed as tentative in that the constructs used in the reassembly studies were not purified from the cell medium, the yields of reassembled Lp(a) were solely based on immunoblot data and the properties of the reassembled products were not provided. Similarly, by incubating LDL with the media from cos-7 cells transfected with apo(a) constructs, Frank *et al.*, (1994a, b) concluded that apo(a) kringle IV-6 is required for Lp(a) reassembly. Moreover, Trieu and McConathy, (1995) utilizing apo(a) constructs partially purified from the media of transfected CHO cells, suggested that both kringle IV-6 and IV-7 can sustain Lp(a) reassembly. It should be noted that none of the cell studies listed above ruled out the possibility that other factor(s) in the cell medium might have influenced the interaction between each apo(a) construct and LDL. On the other hand, the present invention is based on naturally occurring products from human and rhesus monkey sources, and is the first to demonstrate that in their pure form, apo(a) and LDL are competent by themselves to sustain the formation of Lp(a).

The inventors' studies have established that a functional LBS in kringle IV-10 is not directly responsible for Lp(a) reassembly, in that apo(a)s obtained from either the human Lp(a) mutant or rhesus monkey Lp(a), both Lys^-, were as efficient as wild-type human apo(a) in forming an Lp(a) complex. Moreover, the disassembly behavior of the two Lys^- species was comparable to that of native Lys^+ Lp(a). The lack of participation

of apo(a) kringle IV-10 in Lp(a) reassembly has been suggested by Ernst *et al.*, (1995) from the study of apo(a) recombinants. Thus, the lys/pro binding domain plays a dominant role in Lp(a) assembly (see FIG. 5). This would explain why human subjects and rhesus monkeys with a functionally defective LBS in kringle IV-10 are competent to form Lp(a). Collective evidence shows that lys/pro domain spans the apo(a) region between kringle IV-4 and kringle IV-9 including the interkringle linkers. This would be in keeping with the finding that the kringle IV-2 repeats have no affinity for lysine (Li *et al.*, 1992) and are unable to sustain Lp(a) assembly (Ernst *et al.*, 1995; Frank *et al.*, 1994a, b).

These studies have also shown that the apoB100 of VLDL readily affiliates with apo(a) to form a stable complex which can only be dissociated under reducing conditions. This observation establishes that VLDL is competent to form a covalent complex with apo(a) and corroborates the results of previous studies demonstrating the occurrence in human plasma of apo(a) linked to triglyceride-rich lipoprotein particles (Bersot *et al.*, 1986; Scanu *et al.*, 1992; Selinger *et al.*, 1993). These observations also support the notion (Scanu, 1990) that Lp(a) represents a broad class of lipoprotein particles, both cholesteryl ester and triglyceride-rich having as a protein moiety apoB100 linked to apo(a).

One aspect of the present invention was to determine whether a reassembly defect could account for the very low plasma levels of Lp(a) present in the human mutant with kringle IV-10 Arg72 in contrast to the normal levels exhibited by the rhesus monkeys with the same substitution. Behind this hypothesis was the assumption that free apo(a) incompetent to affiliate with the apoB100 of LDL, would be cleared from the plasma at a comparatively higher rate than Lp(a). However, this hypothesis was not supported by the experimental findings. First, the apo(a) present in the plasma of the human mutant was in the form of Lp(a). Second, the free apo(a), obtained by the mild reduction of Lp(a) from either the human mutant or the rhesus monkey, when mixed with either human or rhesus LDL formed an Lp(a) complex with comparable kinetics. Third, the LDL obtained by the reduction of either the human mutant or rhesus Lp(a)

was able to restore an Lp(a) complex when mixed with wild-type free apo(a). These results suggest that the low plasma levels of Lp(a) observed in the human mutant may depend not on a reassembly process but on defective production and/or secretion of apo(a) and that the difference in plasma levels of Lp(a) between human mutant and rhesus monkeys may be attributable to the divergent structural properties demonstrated to exist between the two apo(a) species (Tomlinson *et al.*, 1989).

In summary, it has been shown that apo(a) can be readily dissociated from Lp(a) *in vitro* and that the resulting native apo(a) has the capacity to covalently link again with apoB100 to reconstitute either CE-rich or TG-rich lipoproteins. The domain in apo(a) involved in the reassembly process is spatially removed from kringle IV-10 which is responsible for the binding of Lp(a) to lysine-Sepharose™. These findings, which are based on naturally occurring products, clearly support and extend the notion that Lp(a) particles can be formed extracellularly although do not rule out an intracellular event. Moreover, the readiness whereby apo(a) is released from Lp(a) suggests that the production of apo(a) can occur *in vivo* at sites where reductive conditions prevail. These studies are important for the understanding of the molecular basis for the athero-thrombotic potential of Lp(a).

Another aspect of the invention is the defining of the hydrodynamic and conformational properties of free apo(a). This example illustrates the properties of free apo(a), obtained by the novel methods of Example 1.

EXAMPLE 2

DEFINITION OF THE HYDRODYNAMIC AND CONFORMATIONAL PROPERTIES OF FREE apo(a)

A. MATERIALS AND METHODS

1. Dissociation and Isolation of apo(a) and Lp(a)

Lp(a), 2 mg/ml protein (2 ml), was incubation with DTE at a final concentration of 2 mM in 10 mM Phosphate, 1 mM EDTA, 0.01% NaN_3, pH 7.5 (Buffer A) for 10 min under argon gas at room temperature. EACA to a final concentration of 100 mM was then added in small increments and the reaction mixture, (4 ml) was protected from light with aluminum foil and rotated slowly (7 rpm) in a general purpose rotator at room temperature for 1 h. Subsequently, the incubated mixture was dialyzed against 4 liters of Buffer A containing 100 mM EACA (Buffer B), purged with nitrogen gas. After dialysis, an equal volume of 60% sucrose in Buffer B was added and the resulting mixture distributed into Beckman polycarbonate tubes, product #349622, so that about 0.5 mg of free apo(a) was contained in each tube, placed into a TLA 100.3 titanium rotor and spun in a table top ultracentrifuge (TL100; Beckman Instr., Inc., Fullerton, CA) at 10°C, 100,000 RPM for 18 h at acceleration and deceleration settings of 6. After centrifugation, the top 0.5 ml fraction contained Lp(a) devoid of apo(a) (Lp(a-)) and unreacted Lp(a), and the bottom 1.0 ml fraction contained free apo(a) in pure form. The latter was stored in the sucrose solution at -80°C.

The hydrodynamic studies use a Beckman Optima XLA analytical ultracentrifuge with the analyses being conducted on apo(a) in the absence and in the presence of EACA and proline. The rationale for using these two reagents is that both interfere with the Lp(a) reassembly process and it was necessary to determine whether this would directly affect the structural properties of apo(a). Common hydrodynamic parameters are measured, *i.e.*, sedimentation and diffusion coefficients, viscosity and

Stokes radius. The molecular weight of apo(a) is determined in phosphate buffer at pH 7.2 as a function of apo(a) concentration in order to assess whether apo(a) is monomeric or self-associated in solution. The Stokes radius of the monomer is determined either from the diffusion coefficient or by combining the sedimentation equilibrium with the sedimentation velocity data. In parallel, viscosity measurements may be carried out in a Cannon-Manning Semi-micro ELC 50 viscometer. Based on viscosity values, it is possible to assess the overall conformation of apo(a), *i.e.*, whether the protein is flexible and extended (high intrinsic viscosity between 10 and 30 cm^3/g) or is in a compact and rigid structure similar to globular proteins (intrinsic viscosity between 3.3 and 4.0 cm^3/g). The effect on this conformation by increased concentrations of EACA and L-proline may also be determined.

The secondary structure analyses of apo(a) in the presence and absence of EACA and proline, is carried out on a Jasco J-600 spectropolarimeter (Jasco, Japan) and analyzed using the Jasco-700 software after converting the data by the Softsec File conversion program (Softwood Co., CT). Spectra are recorded at protein concentrations ranging from about 0.1 mg/ml to 2 mg/ml in cuvettes of 0.01 to 0.1 cm path length. Mean residue ellipticities are calculated using a mean residue weight of 112.8 for apo(a). The secondary structure content is calculated by two methods, one using the program VARSLC1 starting with a set of 33 reference proteins (Manavalan and Johnson, 1987), and the other, the program CONTIN (Provencher and Glockner, 1981). All samples are dialyzed against Buffer A with or without EACA or proline but without PMSF (antiproteolytic agent) since this reagent interferes with CD absorption in the far ultraviolet region of the spectrum. The effect of size polymorphism on the hydrodynamic and conformational properties of apo(a) may also be determined.

EXAMPLE 3

FUNCTIONAL AND METABABOLIC DIFFERENCES BETWEEN ELASTASE-GENERATEDFRAGMENTS OF LP(A) AND APO(A)

This example illustrates the effects of elastase enzymes on apo (a) and further relates to the structural and functional properties of fragments from proteolytic cleavage.

A. MATERIALS AND METHODS

1. Isolation and purification of fragments obtained by limited proteolysis of apo(a) and parent Lp(a).

Studies with porcine pancreatic elastase. The choice of porcine elastase, is based on previous studies showing that this enzyme cleaves plasminogen into two lysine binding fragments and a domain called mini plasminogen, acting at the level of the small neutral amino acids located in the interkringle regions (Sottrup-Jensen *et al.*, 1978). Studies have shown that when apo(a) is digested by porcine elastase it generates three fractions, one of which is able to bind to lysine-Sepharose™. This example illustrates the analysis of apo(a) and Lp(a) by comparing the fragments obtained from the elastase digestion of Lp(a) and apo(a) and identifying the specific apo(a) region which interacts with apoB100 and fibrin.

apo(a) is incubated with porcine pancreatic elastase (EC 3.4.21.36) at a molar ratio of 25:1 (apo(a):elastase) in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5 for 2 hr at room temperature. The reaction is terminated by the addition of 5 mM DFP and the product immediately applied to a lysine-Sepharose™ column equilibrated with 10 mM PBS, pH 7.5. The column is then washed with 3 column volumes each of PBS, 0.5 M NaCl and 200 mM of the lysine analogue, EACA. Each eluted fraction is analyzed on Western blots of reduced and non-reduced SDS-PAGE (4-20% polyacrylamide gradient, Novex precast gels) and probed with monospecific antibodies directed against whole

apo(a) and kringle V. In parallel, gels are stained with Coomassie Blue to identify fragments that would not be recognized by the antibodies. Based on the gel profiles, those fractions exhibiting size heterogeneity are purified by molecular sieving chromatography using gel media of appropriate pore size (Sephadex™, Sephacryl™ or Superose™; Pharmacia). All of the fractions are further purified by ion-exchange chromatography using the MonoQ™ column on an FPLC system (Pharmacia) with gradients of 0-1 M NaCl in 10 mM Tris buffer, pH 7.5. The final purity of the fractions is assessed by Western blots of reduced and non-reduced SDS-PAGE as described above.

Lp(a) is digested with porcine elastase as outlined for apo(a). The digest is centrifuged at d 1.21 g/ml in order to separate the floating fractions containing lipids from those which are lipid-free protein (sedimenting fraction). The floating and sedimenting fractions are dialyzed in membranes of 10,000 kDa and 3,500 kDa cutoff, respectively, against PBS. Each fraction is applied to a lysine-Sepharose™ column and the homogeneity of the eluted fractions determined by SDS-PAGE in 4-20% and 3.5% gels (the 3.5% gel is used since some of the fractions will contain an apoB100: apo(a) complex too large to resolve on the 4-20% gradient gel). The fractions are further purified following the procedure described above for apo(a) except that, in addition, the gels are probed with antibodies directed against apoB100.

Studies with thermolysin. In parallel with elastase studies, studies are also performed with thermolysin, which also cleaves at the level of the neutral amino acids. Studies by Huby *et al.* (1994; 1995) have previously shown that digestion of Lp(a) by this enzyme releases fragments identified as the N-and C- terminal portions of the apo(a) in Lp(a). Lp(a) or apo(a) are digested with thermolysin (from *Bacillus thermoproteolyticus*; 40 units/mg of lyophilisate; Sigma) at 37°C for 30 min in 125 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, 0.01% EDTA and 0.01% NaN_3, pH 8.0 at a thermolysin:apo(a) mass ratio of 1:500. The reaction is stopped with 10 mM EDTA, and the purification of the resulting fragments is performed in the same manner as described for the elastase digested apo(a) and Lp(a).

2. Structural and functional characterization of the purified enzymatic digests.

LDL binding. The inventors have previously shown that the incubation of apo(a) with LDL results in Lp(a) particle with properties indistinguishable from those of native Lp(a) (Edelstein *et al.*, 1995). In the current studies, the incubation of F2 with LDL at a molar ratio of 50:1 (LDL:F2) for 5 h at room temperature, produced an F2-LDL covalent complex. This was not the case for F1. The yields of the reassembled complex with F2 were comparable to those obtained when apo(a) was incubated with LDL (Table 6). Similarly, the assembly was inhibited by either EACA or proline. In the unreduced form, the F2-reassembled particle migrated electrophoretically as a single band which was immunostainable by both anti-apo(a) and anti-apoB100. On reduced gels, anti-apo(a) staining revealed a band corresponding to fragment F2; in turn, immunostaining with anti-apoB100 showed one band which migrated in the position of apoB100. From these results the inventors discovered that combining F2 with LDL generated a particle differing from Lp(a) by the absence of the N-terminal fragment, F1. This particle was called R-miniLp(a) to differentiate it from the miniLp(a) which the inventors generated by the cleavage of Lp(a) with elastase. MiniLp(a), obtained from the elastase digestion of Lp(a), exhibited fragments of apoB100. Therefore, in functional studies the inventors utilized R-miniLp(a) particles where the fragmentation of apoB100 was absent. By electron microscopy (FIG. 9), native Lp(a), miniLp(a) and R-miniLp(a) had comparable mean diameters of 24.2 ± 4.2 (n=184), 24.8 ± 3.7 (n=190) and 25.1 ± 3.7 nm (n=202), respectively. However, R-miniLp(a) appeared somewhat more homogeneous in size than native Lp(a) or miniLp(a).

TABLE 6

Reassembly of Apo(a) and the C-Terminal Fragment (F2) with LDL

Incubation mixture	No additive	EACA[a]	L-proline[a]
		% reassembled	
Apo(a) + LDL	70	15	25
F2 + LDL	60	10	20

All incubations were performed using apo(a) or fragment F2 and human LDL at an apoB100:apo(a) molar ratio of 50:1 for 5 h at 37°C. After centrifugation overnight in sucrose at d = 1.127 g/ml, three floating fraction was analyzed for the presence of apoB100:ap(a) complexes by ELISA.

[a]EACA or proline (100 mM) was incubated with apo(a) or fragment F2 for 1 h at 37°C before incubation with LDL.

Binding to lysine-Sepharose™. Like native Lp(a) and apo(a), R-miniLp(a) and F2 bound specifically to lysine-Sepharose™ in that they were eluted from it with EACA. In contrast, F1 representing the N-terminal portion of apo(a) did not bind specifically to the column and was recovered in the PBS flow-through volume.

Fibrinogen binding. F1 failed to bind to immobilized fibrinogen, whereas F2 and R-miniLp(a) exhibited specific and saturable binding (FIG. 10A). The Kd values were in the nanomolar range and were comparable to those of apo(a) and Lp(a) (Table 7). Although F2 and apo(a) exhibited a comparable binding to fibrin in terms of B_{max} values, the binding was significantly decreased when these proteins were components of R-miniLp(a) and Lp(a), respectively (FIG. 10A and Table 7).

TABLE 7

Binding of Lp(a), Apo(a) and the Derived Fragments to Fibrinogen and Fibronectin

Sample	Binding to fibrinogen[a]		Binding to fibronectin[b]	
	Kd[c]	Bmax[c]	Kd	Bmax
	nM	*fmol*	*nM*	*fmol*
Lp(a)	5.8 ± 0.8	1.3 ± 0.8	2.5 ± 1.6	1.8 ± 0.5
R-miniLp(a)	13.3. ± 3.6	6.1 ± 1.3	10.3 ± 1.3	10.9 ± 0.1
Apo(a)	3.8 ± 2.9	36.6 ± 2.9	2.5 ± 2.1	92.0 ± 32.0
F2	10.3 ± 5.1	56.2 ± 32.2	8.8 ± 4.9	23.1 ± 5.3
F1	N/A[d]	N/A	N/A	N/A

[a]EACA-inhibitable, or lysine-mediated binding, obtained by subtracting the binding in the presence of 0.2 M EACA from the total binding.

[b]Total binding, since in the presence of 0.2 M EACA the binding to fibronectin was not significantly affected.

[c]Kd and Bmax were calculated as described in Material and Methods. The values are expressed as mean ± SD from at least three independent studies in duplicate.

[d]N/A - not available, since F1 exhibited no binding to either fibrinogen or fibronectin.

Fibronectin binding. F2 and R-miniLp(a) bound to immobilized fibronectin in a saturable, concentration-dependent manner (FIG. 10B) and exhibited apparent Kds comparable to those obtained with apo(a) and Lp(a) (Table 7). In contrast, F1 failed to bind to fibronectin. of note, EACA did not affect the binding. As in the case of fibrinogen, the B_{max} was significantly decreased when F2 and apo(a) were complexed to LDL (Table 7).

Based on these results, the inventors concluded that F2 contains binding sites for both fibrinogen and fibronectin and that these sites are partially hidden when this fragment associates with LDL.

3. Functional Studies

Reassembly studies: Purified fragments, (1 μg) are incubated with an autologous preparation of LDL in Buffer A at an apoB100:apo(a) fragment molar ratio of 50:1 in a total volume of 175 μl (final fragment concentration, 5.7 μg/ml) in a shaking water bath at 37°C for 5 h in the presence of 50 μM BHT, aprotinin (10,000 KIU/ml) and 1 mM PMSF, under nitrogen. To assess the effect of EACA or proline on the reassembly process, fragments are incubated with 100 mM of EACA or proline for 60 min at 37°C before the addition of LDL and the incubation continued for an additional 6 h. To quantitate the amount of Lp(a) assembled, an aliquot (125 μl) of the reaction mixture is diluted with an equal volume of 60% sucrose in buffer A containing 200 mM EACA and spun in a TLA100 rotor (tube capacity, 250 μl) at 100,000 rpm at 15°C for 18 h using a TL100 ultracentrifuge (Beckman Instruments, CA). The top fraction (105 μl) is removed and quantitated by ELISA designed to measure the apoB100:apo(a) complex. The bottom 100 μl containing free apo(a) is quantitated by a sandwich ELISA specific for apo(a) using affinity-purified polyclonal anti-apo(a) for coating and alkaline phosphatase-conjugated anti-apo(a) for detection. The results are expressed as the percent of the total apo(a) that reassembled to an apoB100:apo(a) complex in 6 h.

Fibrin binding: The binding assays are performed in triplicate according to the procedure of Harpel *et al.* (1989) with some modifications. 96-well plates are incubated with 1 mg/well fibrinogen (Sigma) in Tris buffered saline (TBS) for 2 h at 37°C. After the wells are emptied, 2% BSA in TBS buffer is added to the plates for 1.5 h at 22°C. The wells are washed three times with TBST buffer (TBS buffer complemented with 0.02% Tween™-20) and further treated with 3 ng/well of plasmin (Enzyme Research Laboratories) for 40 min at 37°C. Plasmin is inactivated by incubation of the wells with TBST containing the protease inhibitor *p*-nitrophenyl *p'*-guanidinobenzoate (Sigma) at a

final concentration of 0.1 mmol/L for 20 min at 22°C. After two additional washes with TBST, various concentrations of purified fragments from digested apo(a) and Lp(a) diluted with TBS with or without 200 mM of EACA or L-proline are added and incubated overnight at 22°C. Thereafter, the wells are washed three times with TBST, rabbit anti-apo(a) serum (1:500) added and incubated for 1 h at 22°C. At this time, the wells are washed four times with TBST and the secondary antibody (goat anti-rabbit IgG alkaline phosphatase conjugate (1:2,000; Sigma) is added for 1 h at 22°C. After washing four times with TBST, the colorimetric assay at 405 nm is performed. Dissociation constants (K_d) are derived from the Langmuir equation assuming single-site binding according to Fleury and Angles-Cano (Fleury and Angles-Cano, 1991).

4. Mutagenesis of functional domain(s)

In order to identify the specific amino acids responsible for the functional specificity of a given apo(a) digest, constructs are prepared where amino acids are either deleted or mutated and then expressed in CHO cells and human embryonic kidney 293 cells (HEK). The choice of CHO cells is based on the work by Frank *et al.* (1994; 1994) who expressed in these cells constructs of apo(a) varying in size from 3 to 18 kringle IV repeats. These cells have already been used in the expression of wild-type and mutated (Trp72ÆArg) human kringle IV-10. The HEK 293 cells were utilized by Van der Hoek *et al.* (1994) to express a recombinant form of apo(a) containing 17 kringle IV-like domains. These cells have been grown, and amino acid mutations or substitutions in the purified apo(a) fragments have been based on predictions derived from computer modeling analyses. The available crystallographic coordinates of plasminogen kringles and those on human apo(a) kringle IV-10 are used in this analysis. The modeling studies are performed on a Silicon Graphics Indigo2 station equipped with XZ graphics and 3-D visualization. The software, developed by Biosym Inc., includes InsightII, Homology, Discover and Ligand Design.

Once a given fragment is defined from the structural and functional standpoints, PCR™ amplification using primers derived from the published apo(a) cDNA sequence

(McLean *et al.*, 1987) are used for cloning into the CMV-promoter driven mammalian expression vector pcDNA3 (Invitrogen). The expression plasmids are transfected into CHO or HEK293 cells and selected for neomycin resistance at 400 mg/ml. The expression level of the secreted products is determined by ELISA assays of the conditioned medium using monospecific anti-apo(a) antibodies. The expressed wild-type and mutated fragments are purified by a combination of molecular sieve chromatography and affinity chromatography using an anti-apo(a) Sepharose™ column. The expressed apo(a) fragments are tested for their capacity to bind to either apoB100 or fibrin using the techniques already outlined.

5. Discussion

The inventors' studies have shown that limited proteolysis by pancreatic elastase cleaves human apo(a) at the Lle3520-Leu3521 bond located in the linker between kringles IV-4 and IV-5. Since the same cleavage pattern was obtained with both apo(a) and Lp(a), it is apparent that the elastase-sensitive site is not hindered by the linkage of apo(a) to LDL, suggesting that enzyme site accessibility may depend on the intrinsic properties of the linker between kringles IV-4 and IV-5. It is interesting to note that the cleavage by elastase occurs at the end of the β-structure region where the neutral amino acid Ile appears more exposed and available. The Ile-Leu cutting site is consonant with the known specificity for neutral amino acids by the enzymes of the elastase family (Barrett and McDonald, 1980).

EXAMPLE 4

COMPARISON OF THE METABOLISM OF apo(a) AND PARENT Lp(a)

The site and mechanism of Lp(a) removal from plasma is still unclear although the most recent studies have provided strong evidence for the lack or modest participation of the LDL receptor in the process (Snyder *et al.*, 1992; Snyder *et al.*, 1994; Rader *et al.*, 1994). To date, experimental studies on the metabolic behavior of

native free apo(a) are lacking. With the availability of free apo(a) it is possible now to address this question both in the experimental animal and in man.

1. Studies in mice

The choice of the mouse as the animal model is based on the fact that this a null species in terms of apo(a) and it is easy to manipulate experimentally. Moreover the mouse has been used productively in transgenic studies.

BALB/c male mice are anesthetized with Metafane and injected into the tail vein with 25 mg of either pure apo(a) or Lp(a). Each mouse is under anesthesia at each bleed time point. Seven bleedings, three from the right and three from the left orbital vein and one from the heart are carried out. Bleeds are performed after 1, 15, 30, 120, 180, 300 and 420 minutes. The amount of blood at each interval is 50 μl collected in heparinized hematocrit tubes. The plasma is separated by centrifugation and used for ELISA specific for human apo(a). The inventors carried out ultracentrifugal and gel electrophoretic analyses in order to define whether the injected products are present in the mouse plasma as free or LDL-bound apo(a). The kinetic behavior of the products injected is determined by standard techniques.

The clearance rates of intravenously injected Lp(a) and the derived products were based on ELISA quantitation of mouse plasma collected up to 24 h after injection to insure that more than 95% of the injected material was removed from the circulation. FIG. 11 shows a semi-log plot of the percent of injected dose as a function of time. The slopes of the curves between 3 and 7 h could be separated into three groups: R-miniLp(a) with the slowest clearance, Lp(a) and F2 intermediate and apo(a), the unfractionated apo(a) digest and F1 the fastest. By following the clearance of the unfractionated elastase digest of apo(a) on reduced electrophoretic gels probed with anti-apo(a) the inventors observed that F1 and F2 had different removal rates. Three hours after injection, the bands corresponding to F1 were not longer visible, whereas, F2 was still present up to the 5 h time point. Table 8 lists the plasma half-

times ($T_{1/2}$), calculated from the slope of the linear portion of each decay curve between 4 and 24 h. The apparent $T_{1/2}$ of F1 is shorter than that of F2 (2.9 and 5.0 h, respectively). In turn, the $T_{1/2}$ of apo(a) is identical to that of the unfractionated digested apo(a) (3.7 h).

TABLE 8

Plasma Half-Times of Injected Lp(a), Apo(a) and Fragments in Mice

Sample injected	n[a]	$T_{1/2}$, h
R-miniLp(a)	3	8.3 ± 2.0[b]
Lp(a)	8	5.1 ± 0.8
F2	5	5.0 ± 0.9
Apo(a)	7	3.8 ± 0.6
Apo(a) digest[c]	2	3.7 ± 0.7
F1	5	2.9 ±0.5

Lipoproteins, apolipoproteins, or the fragments purified from digested apo(a) were prepared as outlined above and injected into the tail vein of normal female mice. Plasma decays were followed for 24 h by quantitative ELISA.

[a]Number of mice.

[b]Values are means ± SEM.

[c]Apo(a) was digested with elastase under limited conditions as outlined above. The whole unfractionated digest was dialyzed against 10 mM phosphate buffer, pH 7.2, filter sterilized and injected into the tail vein of mice.

The inventors also examined on anti-apo(a) immunostained blots of reduced 4% gels, the plasma samples taken one hour after injection into the mouse of Lp(a) and derivatives. Compared to the electrophoretic patterns before injection of Lp(a) and apo(a), those after injection contained new bands in addition to the major apo(a) component, suggesting that a degradation process had occurred *in vivo*. It should be noted that the extent of Lp(a) and apo(a) degradation in plasma was less than 5%, and that the majority of the injected material remained intact. The banding pattern of F1

before and after injection was comparable in banding pattern but increased in band intensity. On the other hand, after injection, F2 exhibited an additional faster migrating band designated by an arrow. The inventors next examined the urine of mice 0-5 h after injection of Lp(a) and derivatives by anti-apo(a) immunostained blots of reduced 4-12% gradient gels. The urinary patterns of Lp(a), apo(a) and F1 showed a broad spectrum of bands, three of which were comparable, migrating at 85, 57 and 33 kDa, respectively. In contrast, the urinary pattern after injection of F2, exhibited two major bands migrating in a more narrow size range (70 to 62 kDa). Of interest, by a KV specific ELISA, none of the urine samples contained KV.

In order to rule out artifactual contributions to the formation of fragments, the collected urine samples from injected mice were incubated at 37°C overnight and compared to freshly collected urine kept on ice. Western blot analyses showed no formation of new fragments. Moreover, the mobility of the bands on the gels was unaffected by changing the pH of the urine (pH 3 to 8). No fragments were observed when the urine of a control uninjected mouse was incubated with intact Lp(a) or its derivatives. It is particularly interesting to note that when apo(a) was digested with pancreatic elastase for prolonged periods of time (12-24 h), the fragments observed, on analysis of the digest by SDS-PAGE, were closely comparable to the patterns produced on gel analyses of urine samples obtained from mice injected with apo(a).

The above data point at important differences between the urinary patterns of injected F1 and F2 both in qualitative and quantitative terms. The F1 pattern was characterized by several bands which were observed as early as one hour after injection, and by ELISA, represented less than 0.5% of that injected. In comparison, the F2 pattern was characterized by fewer bands which appears later (5 h) and represented less than 0.5% of the injected material.

2. Studies in man

apo(a) is labeled with ^{131}I and Lp(a-) with ^{125}I according to procedures established in that laboratory. Aliquots of these two radiolabeled products are then injected intravenously into normal volunteers in order to obtain data on the kinetics of these two Lp(a) components.

In a second set of studies, ^{131}I apo(a) and ^{125}I Lp(a-) is first mixed to reconstitute a double-labeled Lp(a) which is then injected intravenously into normal volunteers. These kinetic studies allow the inventors to determine whether Lp(a) is catabolized as a lipoprotein particle or whether apo(a) dissociates and is metabolized independent of the LDL component of Lp(a).

In a third set of studies the normal volunteers are injected intravenously with native Lp(a) labeled with ^{131}I and a reassembled Lp(a) labeled with ^{125}I on apoB of LDL. These data establish whether the kinetics of reconstituted Lp(a) are the same as those of the native Lp(a) which was used to generate apo(a) and LDL. One of the expected difficulties in studying the kinetics of free apo(a) is that once intravenously injected it may rapidly associate with the existing endogenous pool of LDL thus confounding the interpretation of the results. To circumvent this problem parallel studies have been conducted using as recipients, subjects with abetalipoproteinemia in whom the plasma LDL and Lp(a) levels are either absent or extremely low. The protocols used are the same as for normal volunteers.

One of the goals was to define the properties of the Lys/Pro binding domain which becomes fully functional only after apo(a) is freed from its covalent attachment with Lp(a-). By comparing the results of the analytical gels of the purified enzymatic digests from Lp(a) and apo(a) the inventors have been able to readily identify the bands which are uncommon to the two digests and will likely correspond to the apo(a) domain in contact or shielded by apoB100. The precise functional mapping is aided by the mutagenesis studies in which deletions or substitutions of critical amino acids are

carried out. These are important issues in that some therapeutic interventions directed at lowering the plasma Lp(a) levels are based on the undocumented premise that apo(a) is cleared from the circulation at rates higher than Lp(a). This presumed beneficial effect, however, may have to take into account the fact that apo(a) is functionally more active in its free form than in its bound form and that the originally masked site(s) in Lp(a) may become significantly more athero-thrombogenic. Finally, the studies with naturally occurring mutants shed light on the relative importance of the Trp72→ Arg mutation in the lysine binding pocket of apo(a) kringle IV-10, in the lysine/fibrin function of the Lys/Pro domain also in terms of apoB100 interaction.

All subjects studied exhibited similar results in terms of electrophoretic band patterns irrespective of the plasma Lp(a) protein levels and apo(a) size isoform. In addition to intact apo(a), there were at least three other bands which were later recovered in the d 1.21 g/ml bottom fraction. By ELISA, this free apo(a) represented about 5% of the total plasma Lp(a) fraction.

In terms of urine, the pattern of apo(a) fragments was essentially the same as that observed in mice injected with apo(a). As in the mouse, the apo(a) fragments in human urine failed to react against an anti-KV antibody.

3. Discussion

Example 3 above described the functional divergence between the F2 and F1 fragments generated by elastase cleavage of apo (a). An important difference between F1 and F2 was also unveiled by the studies in which mice were injected intravenously with these two fragments. Based on ELISA and gel analyses, the injected F12 had a short residence time in the plasma and was also rapidly excreted in the urine in the form of several fragments. It was interesting to note that the relatively homogeneous F1 after injection into the tail vein of the mouse, appeared in the plasma as distinct 4-5 electrophoretic bands in the size range of 220-135 kDa and as markedly smaller ones in the urine (size range 100-33 kDa). These data show a precursor-product

relationship between injected F1, plasma fragments and urine fragments probably involving an enzymatic basis for these conversions. The enzyme for this conversion is likely to be an elastase since the *in vitro* studies showed that prolonged digestion with elastase can reduce apo(a) to fragments comparable in size to those in the urine, showing that an elastase-like system may be active in the mouse. The relatively large size of apo(a) fragments in the urine also suggests that the mouse kidney plays an active role in their excretion, in accord with the earlier proposal by Mooser *et al.* (1996).

Contrary to F1, injected F2 had a comparatively longer plasma residence time and was excreted in rather minute amounts in the form of fragments which could only be detected in 15-30 fold urine concentrates. The metabolic divergence between F1 and F2 was also documented by the studies in which the whole unfractionated elastase digest of apo(a) was injected into the mouse showing again that most of the rapidly excreted apo(a) urinary fragments were of F1 derivation.

apo(a) fragments of the F1 type were also detected in 3-10 fold urine concentrates of mice injected intravenously with preparations of Lp(a) or apo(a) which had not been previously digested with elastase. Contrary to the relative homogeneity of these materials prior to injection, several bands were also present in the plasma of the injected mice. Taken together, these data show the normal mouse may have an active elastase-like system capable of cleaving Lp(a) or apo(a). This interpretation may appear at variance with the results of the studies by Mooser *et al.* (1996) who injected Lp(a) into normal mice using as port of injection the jugular vein and found no apo(a) fragments in the urine. Based on the current observations, it is possible that those negative results may be attributable to the relation between amount of injected material and degree of urinary concentration not permitting immunodetection of the apo(a) fragments by their assay.

The inventors' data with injected mice provide a useful basis for interpreting the results of their studies in normal human subjects. In these subjects fragments of

apo(a) are spontaneously present in their plasma. By ELISA they represented about 5% of the total plasma Lp(a) protein and were significantly larger than those in the urine. Moreover, the fragments in the urine had the size and band pattern of those seen in the urine of injected mice. The results invite the speculation that in both animal species these fragments might have derived from the action of elastase-like enzymes probably from the formed elements of the blood. Accordingly, both mice and man would have an enzymatic make-up able to digest a small portion of the total apo(a) mass. Even though the mouse is not an apo(a) animal, it produces plasminogen, a five kringle zymogen, which has a high degree of homology with apo(a) (McLean *et al.*, 1987). In this context, elastase is known to cleave plasminogen *in vitro* in a predictable manner (Sottrup-Jensen *et al.*, 1978; Castellino *et al.*, 1990); moreover, recent studies have shown that plasminogen fragments are present in mouse urine (O'Reilly *et al.*, 1994). The latter studies are of interest in that those fragments were found to have angiostatic action.

To sum-up, enzymes of the elastase family cleave apo(a) *in vitro* at a discrete site generating two main fragments with different chemical, functional and metabolic behavior. From the results in mice and man the inventors suggest that these enzymes are involved in the *in vivo* cleavage of apo(a) and its by-products. Such cleavage would be unlikely to occur in the plasma since under physiological conditions the elastase activity in this medium would be inhibited by the action of α1 antitrypsin and β2 macroglobulin (Castellino *et al.*, 1990). Instead, apo(a) proteolysis would be more likely to occur at the level of cell membranes of polymorphonuclear cells, platelets, or macrophages. Accordingly, elastase-dependent apo(a) fragmentation would be expected to occur under pathological conditions, for instance at sites of inflammation involving an active recruitment of polymorphonuclear cells and macrophages. Of interest, the presence of apo(a) fragments has been reported in human atherosclerotic lesions (Hoff *et al.*, 1994). The present studies show that these fragments would be of the F2 type and thus unaffected by the F1-dependent size polymorphism of apo(a). As a corollary to this, F2 would be more pathogenic than F1 from the cardiovascular standpoint.

EXAMPLE 5

EXAMINATION OF THE DETERMINANTS IN Lp(a) ASSEMBLY

The Lp(a) in plasma is known to be polymorphic in size and density. This polymorphism may be contributed by both the size/density of the apoB100-containing lipoprotein particles and by the size of apo(a). In order to determine the relative contribution by each of these two components to Lp(a) assembly, the following studies are performed.

1. Compare LDL and autologous Lp(a-) for the ability to sustain Lp(a) assembly upon incubation with apo(a) of a given size phenotype.

There is reported evidence that the LDL in Lp(a), or Lp(a-), may differ from that of autologous LDL in that Lp(a-) has a higher molecular weight (about 12% higher)), a lower buoyant density and a significant difference in lipid composition (mol/mol), in particular, a higher triglyceride content (Fless *et al.*, 1986). Moreover, Morrisett *et al.* (1993) from *in vivo* studies using heavy isotopes have concluded that apoB100 in Lp(a) derives from a pool that is metabolically different than that of apoB100 in autologous LDL. Thus, it is important to compare LDL dissociated from Lp(a) and autologous LDL, both isolated from the same subject, for their ability to interact with apo(a) and form R-Lp(a).

Lp(a-) is prepared from the disassembly of Lp(a) by mild reduction with DTE and further purified from unreduced Lp(a) by lysine-Sepharose™ chromatography. The Lp(a-) will elute in the unbound fraction. LDL is isolated from the autologous plasma by sequential flotation at d 1.030 - 1.050 g/ml. The purity of the lipoproteins is ascertained by Western blot analyses of SDS-PAGE using mono-specific antibodies against apo(a) and LDL and their chemical composition determined. Comparisons are

made on the percent of the total apo(a) that reassembled with apoB100 after a 6 h incubation and the effect of EACA and proline.

2. Determine the effect of apo(a) size polymorphism on Lp(a) assembly using apo(a) size polymorphs incubated with an LDL of a defined composition and density

Studies have shown that the efficiency of reassembly is dependent on the size of apo(a). In particular, when the 488 and 289 kDa apo(a) phenotypes where compared, it was found that the 289 kDa apo(a) was 1.5 to 2 fold more effective in its interaction with LDL. The inventors would like to pursue this approach by incubating several apo(a) size polymorphs with a single well defined LDL and examine the properties of the R-Lp(a) species formed. For this purpose a number of apo(a) phenotypes ranging in size from 289 to 488 kDa are available.

3. Define whether LDL size/density polymorphs vary in their capacity to bind to apo(a)

Krauss *et al.* (1995) have presented evidence that small, dense LDLs represent a greater risk for atherosclerotic cardiovascular disease than the lighter forms. The intent here is to explore whether dense LDL can be the precursors of dense Lp(a) and whether dense LDLs might have a preference for given apo(a) size isoforms. Subjects are classified as having either light (d 1.026-1.032 g/ml) or dense LDL (d 1.040-1.054). The LDL of some of the typical subjects were used in these studies.

LDL of d 1.019-1.063 g/ml is dialyzed to d 1.04 g/ml in NaBr at 4°C with four solution changes over 24 h and further fractionated as described by Tribble *et al.* (1992). Dialyzed LDL (3.8 ml) is layered over a NaBr solution of d 1.054 g/ml (4.7 ml) in a 13.2 ml Beckman SW 40 rotor centrifuge tube and overlaid with a NaBr solution of d 1.028 g/ml (4.7 ml). The contents are centrifuged to equilibrium at 40,000 rpm for 40 h

at 15°C. All solutions contain 1 mM EDTA and 0.01% NaN_3. After centrifugation, the contents of each tube is collected (0.4 ml fractions) and analyzed with an ISCO gradient fractionator equipped with UV optics and fraction collector. Subfraction densities are determined with a refractometer and the LDL characterized with respect to purity, chemical composition and size. The particle diameters of the subfractions are determined by electron microscopy and by non-denaturing PAGE in 2.5-16% gradient gels (Nichols *et al.*, 1986) using calibrating standards of known diameters.

4. Explore the effect of VLDL density polymorphism and lipolysis by lipoprotein lipase (LpL) on Lp(a) assembly

The inventors (Scanu *et al.*, 1992) and others (Bersot *et al.*, 1986) have reported the presence in the plasma of TG-rich lipoproteins containing apo(a). The inventors have also provided evidence for the preferential association of a high molecular weight apo(a) with these TG-rich particles (Fless *et al.*, 1990). More recently a different interaction efficiency with apo(a) between the apoB100 of LDL and the apoB100 of VLDL has been shown and also observed that VLDL after lipolysis with LpL binds covalently more apo(a) than undigested VLDL. These studies suggested to the inventors that the TG content of apoB100-containing lipoproteins may influence their ability to associate with apo(a), presumably *via* an indirect effect on apoB100 conformation. The hypothesis that the lipid composition of the core may affect the reactivity of the lipoprotein surface for apo(a), comes also from the studies of Steyrer *et al.* (1994) who compared the Lp(a) reassembly capacity of normal LDL and LDL from LCAT deficient subjects. In light of these findings, it is important to explore whether VLDL density heterogeneity and the impoverishment of their TG core by LpL, can affect the capacity of these particles to interact with apo(a).

5. VLDL subfractionation

Plasma from normolipidemic subjects containing Trasylol (10,000 KIU/ml), EDTA (1 mM), NaN_3 (0.01%) and PMSF (1 mM) is centrifuged at d 1.006 g/ml for 20

h at 15°C at 45,000 rpm. The top floating fraction is further fractionated into three subclasses of S_f 100-400, S_f 60-100 and S_f 20-60 by Lindgren's method (Lindgren *et al.*, 1972) and the chemical composition of each subfraction determined. From the studies of Gianturco and Bradley (1986) it is known that surface composition and reactivity of VLDL from hyperlipidemic subjects may differ from that of normolipidemic VLDL.

6. VLDL lipolysis by LpL

LpL are isolated from bovine raw milk and purified by heparin-Sepharose™ chromatography by methods previously established (Chung and Scanu, 1977). The activity of the stock LpL are assayed according to Iverius and Lindquist-Ostland (1986) using a radiolabeled triolein emulsion as substrate.

7. Lipolysis of VLDL

VLDL incubations are carried out in 10 mM Tris-HCl buffer with 150 mM NaCl, pH 7.5 containing 6% fatty acid-poor albumin in covered polypropylene tubes. Lipolysis of VLDL is initiated by adding LpL and the mixture incubated in a shaking thermostatically controlled 37°C water bath for various time periods (0-120 min). The reaction is terminated by inactivating the enzyme with 2 mM diethyl-*p*-nitrophenyl phosphate (E600) (Williams *et al.*, 1992). The extent of lipolysis is monitored by measuring the levels of free fatty acids before and after incubation using the NEFA C test kit from Wako Chemicals, Inc. Dallas, TX. This enzymatic test is insensitive to glycerol, is highly reproducible, rapid (1 h) and detects as little as 0.25 mEq fatty acid/L. The lipolyzed VLDL is then separated from any unreacted VLDL and albumin by flotation as described above and the composition determined.

Reassembly studies are conducted as described except that VLDL of a defined density, before and after lipolysis, replaces LDL in the reaction tube. Different apo(a) size phenotypes are used in the reassembly reaction.

EXAMPLE 6

CONFORMATION AND STRUCTURAL PROPERTIES OF Lp(a) AS A FUNCTION OF apo(a) SIZE

In a continuing effort to define the effect of apo(a) heterogeneity on the structural and functional properties of Lp(a), the inventors initiated a study of the hydrodynamics of Lp(a). To this end the inventors measured the sedimentation coefficient, partial specific volume, molecular weight and in some instances the diffusion coefficient of homogenous Lp(a) species with defined apo(a) mass is thought to contribute to Lp(a) density, which in turn has a direct effect on the sedimentation rate of Lp(a) in a centrifugal field, the inventors measured the partial specific volume (which is the reciprocal of the Lp(a) particle buoyant density). This allowed correction of the sedimentation coefficient for the buoyancy of the Lp(a) particles and normalization to standard conditions of water and 20°C. Knowledge of the molecular weight as determined by sedimentation equilibrium and the sedimentation coefficient obtained from sedimentation velocity studies allows the calculation of the translation frictional coefficient. The latter is a parameter that depends on the size, shape and flexibility of the Lp(a) molecule. The frictional coefficient is usually expressed as a ratio, *e.g.*, f/fo, and is a measure of asymmetry and hydration. The latter was approximated fro the known hydration of LDL, and that of apo(a), which was calculated form its amino acid and carbohydrate compositions.

Since the sedimentation coefficient is extremely sensitive to changes in shape or conformation, the inventors were also interested to determine whether the binding of the lysine analog, e-amino caproic acid, could effect a change in the conformation of Lp(a). The inventors were encouraged to undertake this investigation by the fact that plasminogen (which has extensive homology with apo(a) undergoes an extremely large conformational change that is induced by the occupation of a weak lysine binding site by ε-aminocaproic acid among other lysine analogs.

Results indicate that $S°_{20,w}$ values of Lp(a) are strongly correlated with apo(a) mass and obey the relation $S°_{20,w} = 7.1 \div 0.37x$ (R = 0.96) where x equals the number of kringle 4 domains in apo(a). When x equals zero, the sedimentation coefficient is 7.1 which is a value representative of a typical LDL molecule. In contrast, Lp(a) mass does not correlate with apo(a) mass or kringle number. Frictional ratios *i.e.*, f/fo values were calculated for each Lp(a) phenotype assuming a hydration of 0.37 g H_2O / g Lp(a). As with Lp(a) molecular weight, f/fo values did not correlate with apo(a) mass or kringle number. The mean f/fo of six different Lp(a) phenotypes was calculated to be 1.23 ± 0.05. In contrast the mean f/fo of two LDL preparations that were assumed to be hydrated to the extent of 0.34 g H_2O / g LDL was 1.01. As expected, a f/fo value of 1 for LDL indicates that this molecule is spherical. The fact that the frictional ratio of Lp(a) in 0.15M NaCl deviates significantly from unity at first hand appears to indicate that Lp(a) is aspherical or even ellipsoid. Axial ratios of ellipsoids calculated from Perrin's formulas appear to indicate axial ratios of 4.5 to 5 for either a prolate or an oblate ellipsoid. These values clearly are not compatible with conclusions derived from electron microscopy and small angle X-ray scattering that appear to indicate that Lp(a) is grossly spherical. Two factors that could account for the deviation from unity may be 1) an underestimation of the hydration of Lp(a), and 2), that in contrast to LDL the surface of Lp(a) may have a higher rugosity. Thus instead of having a smooth, rigid and impermeable surface, the presence of apo(a) girdling the Lp(a) molecule like a like string of beads would provide frictional resistance due to protrusion of individual kringles.

The inventors' studies into the effect of EACA on the conformation of Lp(a) has shown that this lysine analog elicits a severe retardation of the Lp(a) sedimentation rate. The reduction in the magnitude of the sedimentation coefficient is proportional to the concentration of EACA. Assuming that *n* EACA molecules bind to Lp(a) at equivalent binding sites the inventors were able to fit plots of sedimentation rate vs. EACA concentration and derive the dissociation constant and number of binding sites. The mean dissociation constant was 3.7×10^{-10} and the mean number of EACA molecules bound was 4.8. The magnitude of the change in the sedimentation rate was directly

proportional to the mass of apo(a) or number of kringle 4 domains. For Lp(a) phenotypes with large apo(a) polymorphs, this change represented almost a 50% decrease in the sedimentation rate. Compared to this decrease, the 15% decrease in sedimentation rate that plasminogen undergoes on binding EACA is relatively small. Thus magnitude of the conformational change observed with Lp(a) has to be considered massive since plasminogen held the previous record for the largest ligand-induced conformational change.

The inventors also calculated the frictional ratio of Lp(a) from the minimum sedimentation rates achieved at maximal EACA concentrations. These ratios were directly proportional to apo(a) kringle 4 number and had a regression coefficient of 0.996. This was in contrast to the native or compact form of Lp(a) which did not exhibit a similar correlation. Assuming a hydration of 0.37 g H_2O / g Lp(a), frictional ratios of Lp(a) increased in magnitude linearly from 1.4 to 2.1 for particles with the smallest to the largest apo(a) polymorph. Thus, in contrast to native Lp(a), where apo(a) appears to be anchored to the lipoprotein surface, the binding of the ligand EACA by Lp(a) releases most of the apo(a) molecule from multiple anchoring points and favors its extension into the medium where it provides much greater frictional resistance.

A. DETERMINATION OF apo(a) MOLECULAR WEIGHTS BY SDS-GEL ELECTROPHORESIS

SDS-gel electrophoresis is used routinely in the characterization of apo(a) polymorphs, and apo(a) molecular weights are frequently obtained with the use of apoB-100 in addition to high molecular weight standards from either BioRad or Pharmacia. This presents certain problems in that the highest molecular weight standard available from these companies is myosin with a M_r of 200,000, which if apoB-100 is not used, leads to a long extrapolation and therefore considerable uncertainty in the derived molecular weights. Using apoB100 as a standard is also problematic, as its mobility on polyacrylamide gels is anomalously fast, leading to apo(a) molecular

weights that are exaggerated. These problems in the determination of molecular weights by SDS-PAGE. Because the inventors were able to investigate the utility of SDS gel electrophoresis in the estimation of the molecular weights of different apo(a) polymorphs.

Pharmacia 4-15% acrylamide Phast™ gels, Isolab 2.5-16% polyacrylamide gels, 2.5-6% homemade polyacrylamide gels, 4% acrylamide Laemmli gels and 1.5% agarose submarine gels were evaluated. To calibrate the gels the inventors used LDL-apoB100, cross-linked phosphorylase B, and Pharmacia high M_r standards, in addition to the four apo(a) polymorphs whose M_r ranged from 289-488,000. LDL was not useful as a standard because apoB molecular weight was underestimated 30-50% by all acrylamide systems and its inclusion would have exaggerated apo(a) mass up to 60%. Two of the gradient gel systems (4-15% and 2.5-16%) gave apo(a) molecular weights within 10% of their actual values, but were not able to resolve crosslinked phosphorylase B bands greater than pentamers thus limiting their use to apo(a)'s with M_r lower than 500,000. To obtain better resolution of the higher molecular weight phosphorylase B polymers, the use of homemade 2.5-6% acrylamide gels was investigated. These gave slightly better resolution (up to the hexamer of phosphorylase B), but gave less accurate apo(a) molecular weights in that they were overestimated by 30%, while that of apoB was still underestimated by 50%.

The inventors also investigated 4% acrylamide gels using the Laemmli buffer system because of their wide use in the field, although the inventors were aware that the crosslinked phosphorylase B standards were not compatible with this system. The inventors found that the gels had to be overloaded with phosphorylase B for band visualization. These gels also overestimated the molecular weight of apo(a) by 20-25%, and underestimated that of apoB by 40%.

Agarose gels using SDS, TRIS-borate buffer systems gave by far the best resolution (up to heptamer with phosphorylase B), the right molecular weight for apoB, but overestimated apo(a) molecular weights by 60-70%. Because borate is known to

form complexes with carbohydrate, the inventors considered the possibility that this interaction was responsible for the retarded mobility of apo(a). However use of the Weber/Osborn buffer system led to even greater overestimation of apo(a) molecular weights. The inventors are now investigating whether agarose gels with no measurable electro-endosmosis will give more representative apo(a) molecular weights. The inventors conclude that correct molecular weights for apo(a)'s with mass less than 500,000 daltons can be measured with polyacrylamide gradient gels when appropriate standards are used. This is true despite the heavy glycosylation of apo(a).

EXAMPLE 7

STRUCTURES AND FUNCTIONS OF FREE AND apoB100-BOUND apo(a)

A. REMODELING OF Lp(a) IN WHOLE PLASMA BY LPL

The effect of LpL on normotriglyceridemic plasma with two apo(a) size isoforms has been studied. Purified bovine milk LpL obtained from Dr. P. Iverius (Utah), specific activity, 500 μmoles FA/min/mg protein, was incubated with whole plasma at 37°C for 1 hr in the presence of 5% BSA and 0.03% HDL as a source of apoCII. The products were then separated by density gradient ultracentrifugation. Compared to the control plasma, the Lp(A) absorption peak shifted to a higher density and became sharper, also documented by Western blot analyses in SDS-PAGE gels. In addition, LDL became denser while the HDL species moved into a less dense region. The results indicated that Lp(a) can undergo structural changes by the action of LpL and suggest that this enzyme can be a factor in the generation of CE-rich Lp(a). Based on the results in the whole plasma and to better define the products of Lp(a) lipolysis the inventors examined Lp(a) particles of different densities isolated from the plasma of the same subjects.

B. REMODELING OF Lp(a) PARTICLES DIFFERING IN DENSITY

The inventors focused their studies on the Lp(a) isolated from the plasma of subject KB exhibiting two apo(a) alleles (70 and 90 kb), determined by pulse field gel electrophoresis and two apo(a) isoforms (289 and 488 kDa). The Lp(a) species were isolated by lysine-Sepharose™ and then repurified by density gradient ultracentrifugation. In each case the inventors observed a broad density profile spanning from d 1.030 to 1.090 g/ml with three main components of average peak density of 1.032, 1.052 and 1.078 g/ml. The first two fractions contained the 289 kDa isoform and the third, more dense, the 488 kDa isoform. Each of Lp(a) species were subjected to the action of LpL.

C. EFFECT OF LpL ON Lp(a), d=1.032 g/ml

The low density Lp(a) fraction (d=1.032 g/ml) containing a higher TG/CE ratio than classic Lp(a) and only one apo(a) isoform (289 kDa), was hydrolyzed with LpL essentially following the protocol for whole plasma except that after the first hour of lipolysis, the Lp(a) sample was exposed to additional fresh enzyme for an additional hour. The products were separated by ultracentrifugation in a performed density gradient, 0-12% NaBr, the pertinent fractions combined and examined for their chemical composition and also by SDS-PAGE followed by Western blotting using a mono-specific Lp(a) antibody. The absorbence profile showed that as compared to the untreated Lp(a), the major peak of the LpL-treated Lp(a) shifted from d1.032 to 2.036 g/ml. Chemical analysis of the fractions before and after lipolysis demonstrated that the TG content was reduced by 32% while the cholesteryl esters, free cholesterol and phospholipids remained essentially unchanged. The Western blots exhibited a normal isoform pattern with no evidence of apo(a) degradation. For comparison, the LDL fraction from the same subject was isolated and also treated with LpL under the same conditions. After lipolysis, LDL became slightly denser with some narrowing of the density peak. The TG content decreased by only 10% while the rest of the lipid composition was unchanged.

D. EFFECT OF LpL ON Lp(a) OF d=1.050g/ml

This Lp(a) fraction contained one apo(a) isoform of 289 kDa and banded at d=1.050 g/ml. When subjected to LpL hydrolysis followed by density gradient ultracentrifugationthe peak became sharper and banded in a more dense region, d=1.055 g/ml. Table 5 lists the chemical composition of this Lp(a) species before and after lipolysis. Most significantly, the TG content was reduced by 30% while the remaining lipids were unaffected.

TABLE 5

Chemical Composition of Lp(a) Before and After Lipolysis with Lipoprotein Lipase

	% w/w				
Sample	P	PL	CE	FC	TG
-LpL	29.8	15.9	37.8	9.2	7.3
+LpL	31.8	16.1	37.3	9.9	4.9

E. EFFECT OF LpL ON Lp(a) OF d=1.080 g/ml

This Lp(a) particle had the 488 kDa isoform and was the most dense of the particles that the inventors examined. Upon sample lipolysis the density gradient ultracentrifugal profile showed a peak shift to a higher density without changes in peak width.

The inventors' studies indicate that Lp(a) heterogeneity is any partially controlled by the apo(a) gene and that the lipids, and in particular the core TG, are important contributors to lipoprotein density. The inventors' studies also indicate that LpL is a main modulator of the TG-rich Lp(a) as is the case for apoB100-containing lipoproteins (VLDL, IDL and light LDL) suggesting that at least some of the LpL

species in the plasma are generated *via* the lipolytic conversion of TG-rich Lp(a) to CE-Lp(a). The functional properties of these various Lp(a) species and clarification.

F. PREPARATION OF NATIVE FREE apo(a) BY MILD REDUCTIVE CLEAVAGE OF Lp(a)

The inventors have been able to prepare *in vitro* a functionally competent apo(a) starting from human Lp(a) preparations of a defined size, density, composition and apo(a) phenotype. On the premise that the disulfide between apoB100 and apo(a) is more accessible to cleavage that the three intrachain disulfides that stabilize each apo(a) kringle, the inventors subjected Lp(a) purified from plasma to reduction with 2 mM dithiothreitol (DTE), in the presence of 100 mM of the lysine analog ε-aminocaproic acid (EACA). The dissociated apo(a) isolated by sedimentation in a sucrose solution of d=1.127 g/ml, retained its binding capacity for lysine-Sepharose™ and also interacted with cholesteryl ester (CE)-rich, low density lipoproteins (LDL) to restore a complex, CE-Lp(a), which was indistinguishable from the parent Lp(a) in chemical conformation (circular dichroism) and size (electron microscopy).

G. PROPERTIES OF ISOLATED apo(a)

Western blots of SDS-PAGE gels of the dissociated products indicated only free apo(a) was detected in the sedimenting fraction as shown by the absence of apoB containing particles. In turn, the floating fraction contained apoB100 as Lp(a-) and small quantities of unreacted Lp(a). These Western blots also showed that reduction with 3% β-Me causes the free apo(a) band of each phenotype, 488 or 289 kDa, to shift to a higher position in the gel and corresponded to that of the parent reduced Lp(a). Moreover, the phenotypic pattern of the isolated free apo(a) in terms of isoform number and gel mobility resembled that of the parent Lp(a).

Based on amino acid analysis, apo(a) contained 3±2 (n=7) moles of cysteine per mole of protein (289 kDa isoform) as compared to 130±7 (n=7) for the fully reduced

and alkylated product. The theoretical number of fully reduced cysteines was calculated to be 139. The isolated apo(a) examined by far-ultraviolet CD spectroscopy gave a spectrum characterized by a strong negative band at 203 nm and a positive band at 222-232 nm. The analysis of the spectrum by the CONTIN and VARSLCI methods indicated 0-2% α-helix, 66% β-structure and the remainder 32% in mainly random conformation. The isolated apo(a) bound to a lysine-Sepharose™ column and could be eluted from it with 200 mN EACA but not with PBS or 0.5 M NaCl, a behavior similar to that of the parent Lp(a).

H. REASSEMBLY OF Lp(a) FROM apo(a) AND LDL

apo(a), of a defined phenotype was incubated with an homologous preparation of LDL at 37°C. The amount of Lp(a) formed was quantitated by ELISA designed to measure the apoB100:apo(a) complex. Western blots probed with antibodies directed against apo(a) and apoB100, showed that the band corresponding to the reassembled product contained both apoB100 and apo(a), even though the sample had been boiled in SDS prior to gel electrophoresis, suggesting a covalent association between apoB100 and apo(a). This was corroborated by the observation that samples reduced with 3% β-ME, dissociated into bands corresponding to free apo(a) and apoB100 further suggesting that the covalent linkage between these two proteins was *via* a disulfide bond. The percent of apo(a) which was reassembled into Lp(a) was a function of both time of incubation and apoB100:apo(a) weight ratios. At a weight ratio of 100:1, the curve reached a plateau after 8 h at which time 80% of the initial apo(a) mass was associated with apoB100. On the other hand, at a weight ratio of 25:1 a longer reaction time (24 hrs.) was necessary to reach a similar level of reassembly. The potential effect of the initial apo(a) concentration (5.7, 11.4, 28.6 and 45.9 μg/ml) on Lp(a) reassembly was also examined at an apoB100:apo(a) weight ratio of 25:1. No significant differences in results were obtained.

The reassembly of Lp(a) was hampered by the presence of high molarity (100 mM) solutions of either EACA or proline. The inhibitory effect was dose dependent

until reaching the 500 mM concentration, when there was an almost 90% level of inhibition. At concentrations equal to or below 200 mM, EACA appeared to be a relatively more potent inhibitor than proline. On the premise that EACA and proline interact at different sites on apo(a), the inventors incubated apo(a) with 100 mM each of EACA and proline prior to incubation with LDL. Under these conditions the inventors obtained a two-fold inhibition of the Lp(a) reassembly, as compared to the incubation with a single inhibitor.

The inhibitory action of EACA on Lp(a) reassembly suggested an involvement of lysine residues. Thus, the inventors modified the lysines on apoB100 with the acylating reagent citraconic anhydride and incubated the modified LDL with apo(a). Based on ELISA quantitation, only 15% of the total apo(a) was complexed to apoB100 and on Western blots most of the apo(a) was unbound. In turn, none of these conditions significantly affected the stability of either the already reassembled or native Lp(a).

I. REASSEMBLY OF Lp(a) FROM apo(a) AND VLDL

The inventors have previously shown that an apo(a) linked to apoB100 can be found in triglyceride-rich particles isolated from hyperlipidemic plasma. In the current study the inventors examined the *in vitro* interaction of apo(a) with a single apo(a) isoform (289 kDa) with preparation of VLDL isolated from the plasma of two hypertriglyceridemic subjects with type IV dyslipoproteinemia and very low levels of plasma Lp(a) protein, *i.e.*, 0.1 and 0.3 mg/dl and TG levels of 300 and 277 mg/dl, respectively. The experimental conditions for the reassembly were as described previously using a 100:1 VLDL apoB100:apo(a) weight ratio. In the early phase (up to 2.5 h) the reassembly process between apo(a) and VLDL followed a course which was similar to that observed between apo(a) and LDL, however, VLDL required 16 h to reach the 80% reassembly level achieved by LDL in 8 h. The reassembly of apo(a) with VLDL was also inhibited by 100 mM EACA or proline although the inhibitory action of these agents appeared to be more efficient than that observed with the interaction between apo(a) and LDL. Moreover, like the reassembled product from apo(a) and

LDL, the one obtained from the interaction between apo(a) and VLDL was also only dissociable by 2 mM DTE, indicating that a disulfide linkage had been formed.

J. PROPERTIES OF Lp(a) REASSEMBLED FROM apo(a) AND LDL

By isopycnic density gradient ultracentrifugation the reassembled Lp(a), rLp(a), was clearly separated from LDL and banded in the same position as the parent Lp(a). Regardless of the procedure of isolation, rLp(a) bound to lysine-Sepharose™ and was eluted from it with 0.2M EACA in a manner comparable to native Lp(a). By electron microscopy, the rLp(a) particles were slightly heterogeneous with an average diameter of 27.9 nm ± 4.0 S.D. (n=15) very similar to their native counterparts (average diameter, 28.4 nm ± 4.1 S.D. (n=15). The LDL utilized for the reassembly showed a similar heterogeneity with an average diameter of 27.5 nm ± 3.5 S.D. (n=130). On GGE, rLp(a) had the same mobility as control Lp(a) indicating that both had a similar size or Stoke's radius. Moreover, a measured by circular dichroism, the conformation of rLp(a) closely resembled that of native Lp(a). The spectra of both lipoproteins were characterized by negative bands at 218 nm and 210 nm Secondary structure calculations performed using the programs VARSLCI and CONTIN gave a 24% α-helix, 29% β-sheet and 46% random structure for Lp(a). The chemical composition of the rLp(a) was also comparable to that of the parent Lp(a) and had the same lipid composition as the LDL preparation used in the reassembly system.

The inventors also assessed the effect of EACA and proline on the stability of rLp(a). After the reassembled product was formed, it was incubated with 100 mM of either EACA or proline for 1 h at room temperature. The mixture was then centrifuged at d=1.127 g/ml for 18 h and the floating fraction quantitated for apoB100:apo(a) complexes by ELISA. Addition of either EACA or proline caused some apo(a) dissociation (10-12% of the total rLp(a), indicating that a small component of apo(a) escaped covalent linking to apoB100 and co-purified with the stable complex, a conclusion supported by the results with 2 mM DTE. Addition of this reducing agent to rLp(a) promoted almost complete dissociation of the apoB100:apo(a) complex.

The results of these studies showed that apo(a) can be dissociated from Lp(a) under mild reducing conditions and that the free apo(a) so obtained is "native" in that it is still able to bind to lysine-Sepharose™ and to reassemble into lipoproteins containing apoB100. The initial phase of the reassembly process appeared to involve non-covalent interactions followed by a stabilizing step in which apo(a) forms a disulfide bond with apoB100. The reassembly process appeared to be independent of the nature of the core lipids of Lp(a) in that both CE-Lp(a) and TG-Lp(a) particles can be readily generated.

K. DOMAINS IN FREE apo(a) INVOLVED IN THE BINDING TO LYSINE-SEPHAROSE™, PM-FIBRIN AND IN THE REASSEMBLY WITH LDL

The inventors have previously identified human mutants having a Lys^- Lp(a) and Arg72 of Trp72 (Lys^+, wild-type) in the LBS of kringle IV-10. In order to assess the role of the LBS in lysine and fibrin binding the inventors have compared the behavior of Lp(a) and apo(a) (obtained by mild reduction as described above) from human wild-type (WT) and mutant (M) subjects with respect to their binding to lysine-Sepharose™ and PM-fibrinogen. Lysine-Sepharose™ affinity chromatography showed that contrary to WT, the Lp(a) from the M subject failed to bind. However, upon exposure to the detergent 0.1% Tween®, M Lp(a) bound to the column and could only be eluted with EACA. On the other hand, both WT and M Lp(a) and their respective apo(a)s exhibited binding (K_d for WT Lp(a) and apo(a), 35 and 87 nM, respectively; K_d for M Lp(a) and apo(a), 99 and 33 nM respectively) which was lysine and proline dependent. Moreover, incubation of wild-type and mutant free apo(a) with their autologous LDL, generated Lp(a) complexes which were structurally and functionally indistinguishable from their parent native Lp(a). The same results were obtained by using LDL preparations freed of apo(a), Lp(a-), by the treatment of Lp(a) with DTE. In each instance the reassembly process was inhibited by the presence of either EACA or L-proline with a 2-fold increase in the efficiently of the inhibitor when the two reagents were used together.

These two reagents had a minimal effect on either Lp(a) or reassembled Lp(a). These results showed that: (1) regardless of the source of Lp(a), *i.e.*, Lys^+ or Lys^-, all of the isolated free apo(a)s were Lys^-; (2) disassembly of Lp(a) into free apo(a) and Lp(a-), uncovers a domain on apo(a) which is both EACA and proline sensitive; (3) the mechanism of binding of Lp(a) to lysine-Sepharose™ is distinct from that of PM-fibrin; and (4) the presence of Arg72 in the LBS of kringle IV-10 is not involved, at least directly, in the assembly process.

L. LIMITED PROTEOLYSIS OF apo(a) BY PORCINE PANCREATIC ELASTASE

Lp(a) and apo(a) were digested with pancreatic elastase under conditions of limited proteolysis as described. Lp(a) and apo(a) containing a single phenotype were incubated with porcine pancreatic elastase in 50 mM Tris-HCl containing 100 mM NaCl, pH 8.0. The apo(a):elastase ratio was 50:1 (mol/mol). The digestion was allowed to proceed for 5 h at room temperature with gentle stirring. The reaction was terminated by the addition of diisopropylfluorophosphate(DF) to 5 mM and incubated for an additional 30 min at room temperature. The mixture was applied to a lysine-Sepharose™ column previously equilibrated with 10 mM phosphate buffer containing 100 mM NaCl, pH 7.5. The column was then washed with 3 column volumes of equilibration buffer followed by three column volumes of phosphate buffer containing 500 mM NaCl and finally with 200 mM EACA. The Lp(a) and apo (a) digests were subjected to 4% SDS-PAGE immunoblot analysis using a rabbit anti-apo(a) polyclonal antibody.

Immunostained gels were run under unreduced (left) and reduced (right) conditions. In the unreduced gels, digested Lp(a) and apo(a) exhibited a common migrating band, designated as F1. On reduced gels, the banding patterns of digested Lp(a) and apo(a) were identical. F1 had an apparent mass of 220 kDa and was associated with a second band with an apparent mass of 170 kDa, designated F2, and

a set of faint ones differing in size by approximately 20 kDa, which is the apparent size of a single kringle. On reduced gels, apo(a), F1, F2 and the repeating bands migrated with slower mobilities than under nonreduced conditions, likely due to the effect of the reducing agent on the conformation of each product. The limited proteolysis of Lp(a) and apo(a) were also carried out by another enzyme of the elastase family, human leukocyte elastase. On anti-apo(a) immunostained reduced 4-12% gradient gels, the banding pattern of the leukocyte elastase digest was comparable to that of the digest from pancreatic elastase. Leukocyte elastase digestion of Lp(a) and apo(a) and subsequent isolation of the proteolytic products gave comparable results to those obtained with pancreatic elastase. Based on these results, in subsequent studies pancreatic elastase was utilized since this enzyme was readily available in a highly purified form and in relatively large quantities.

1. Ultracentrifugal separation of the products obtained by digestion of Lp(a) with pancreatic elastase

Digested Lp(a) was diluted 1:1 (v/v) with 60% sucrose in buffer A containing 200 mM EACA to a final density of 1.127 g/ml and the mixture centrifuged in a TL 100 tabletop ultracentrifuge at 15°C, overnight. Two fractions were collected; a top, (0.5ml), and a sedimenting one (1 ml). In the anti-apo(a) immunostained unreduced gels, the top fraction contained a band representing undigested Lp(a) and a second one which migrated faster than Lp(a). Both bands were also detected with an anti-apoB100 antibody. Upon reduction, these bands disappeared and were replaced by a faint apo(a) band and one migrating in the position of F2. The latter was unreactive to the anti-apoB100 antibody. The bottom fraction contained F1 and a few faint ones with a faster migration. In addition, the bottom fraction was unreactive to anti-apoB100 antibody and based on a specific ELISA, did not contain KV.

The d 1.127 g/ml top was treated with 1.5 mM DTE in the presence of 100 mM EACA and centrifuged in 30% sucrose according to the method utilized by the inventors for the isolation of free apo(a) (Edelstein *et al.*, 1995). The sedimenting

lipid-free fraction contained a small amount of undigested apo(a) and F2. The latter was purified by molecular sieving on Superose 6. By electrophoresis, the isolated F2 exhibited a band with an apparent mass of 170 kDa, as detected by an anti-apo(a) antibody. Based on specific ELISAs, F2 contained KV but apoB100. Thus, the limited proteolysis of Lp(a) by elastase produced two fractions: one of them, referred to as F1, was lipid-free, sedimented at d 1.127 g/ml, and reacted against anti-apo(a) but not against anti-KV or anti-aopB100; the second one, was lipid-rich, floated at d 1.127 g/ml and reacted against anti-apoB100, anti-apo(a) and anti-KV. Mild reduction of this fraction generated a 170 kDa band, F2, reactive against anti-apo(a) and anti-KV and non reactive against anti-apoB100. The inventors interpreted the above results to indicate that the d 1.127 g/ml floating fraction is a lipoprotein particle containing apoB100 covalently linked to F2. The inventors called this lipoprotein, miniLp(a). This nomenclature was recently proposed by Huby *et al.* (1995) to designate a lipoprotein particle which they obtained by digesting Lp(a) with thermolysin. In those studies thermolysin caused hydrolysis of apoB100. In the current studies elastase also caused a partial cleavage of apoB100 in Lp(a) and the resulting fragments remained associated with the lipoprotein particle (see Section on Functional studies).

Thus, limited elastase digestion cleaves Lp(a) into a lipid-free fraction, F1 and a miniLp(a) particle in which F2 is linked covalently to LDL containing a partially hydrolyzed apoB100.

2. Isolation by lysine-Sepharose™ chromatography of the products obtained by limited digestion of apo(a) with pancreatic elastase

Free apo(a) digested with pancreatic elastase was applied to a lysine-Sepharose™ affinity column which was then washed with three column volumes of PBS, 500 mM NaCl and 200 mM EACA (**FIG. 6**). Two major peaks were observed, one eluting with PBS and one with EACA. Electrophoretic analyses on reduced gels (4-12%) probed with anti-apo(a), showed that the unbound fraction eluting with PBS,

represented F1 migrating in the 220 kDa position and the fraction, eluting with EACA represented F2, migrating in the 170 kDa position . Of the two fractions, only F2 reacted against anti-KV.

3. Amino terminal sequence analyses of the proteolytic fragments obtained from digested Lp(a) and apo(a)

FIG. 7 shows the partial NH_2-terminal sequences of F1 and F2 obtained from the elastase digestion of Lp(a) and apo(a). By aligning these sequences with those of apo(a), using the program ClustalW, the inventors located the cleavage site at the Ile3520-Leu3521 bond in the linker region between kringles IV-4 and IV-5. From these data the inventors concluded that the apo(a) fragment which eluted from the lysine-Sepharose™ column with PBS (**FIG. 6**) was identical to the ultracentrifugal d 1.227 g/ml bottom fraction and corresponded to the N-terminal portion of apo(a) comprising KIV-1 through KIV-4. In turn, the fraction which eluted with EACA (**FIG. 6**) was identical to F2, the apo(a) fragment obtained by mild reduction of elastase digestion Lp(a). This fraction corresponds to the C-terminal fragment of apo(a) containing kringles IV-5 through IV-10, kringle V and the protease region. The electrophoretic migration of F1 varied according to apo(a) size, an indication of its dependence on the number of KIV-2 repeats. In contrast, F2 exhibited a constant size which, based on amino acid composition, the inventors calculated to be 113,030.

Thus, whether starting from Lp(a) or free apo(a), elastase cleavage produces two main apo(a) fragments, F1 and F2, representing the N- and C-terminal components of apo(a), respectively. A schematic diagram of the structure of Lp(a) and the derived fragments is shown in **FIG. 8**.

M. METABOLISM OF apo(a) AND Lp(a) IN NORMOLIPIDEMIC MICE

BALB/c mice were injected in the tail vein with 25 μg of apo(a) or Lp(a) in a total volume of 200 μl and bled at timed intervals from the orbital vein. Mouse plasma was analyzed for the levels of apo(a) or Lp(a) by a sandwich ELISA using mono-specific antibodies directed against apo(a), Lp(a) and human apoB100. Between 120 and 420 min after the initial injection the slope of the decay curve was linear. The $T_{1/2}$ value for this interval was significantly higher (10.5 h) for Lp(a) than for apo(a) (1.65 h).

The structural consequences of mutations in apo(a) kringle IV-10 have been studied by computerized molecular modeling. The inventors have investigated two mutations which were observed in the kringle IV-10 of apo(a).

N. SUBSTITUTION OF Trp72 BY Arg72

This mutation was found in a subject whose Lp(a) exhibited a lack of lysine binding. The mutation was localized to the lysine-binding pocket of the kringle. The lysine-binding pocket consists of a hydrophobic V-shaped trough which has the form of an elongated, open shallow depression located at the kringle surface. The depression is lined by aromatic rings Phe-64, Trp-62 and Trp-72, the latter two oriented in an antiparallel manner to each other. The anionic (Asp-55/Asp-57) and cationic (Lys-35/Arg-71) charge pairs at either end of the trough from a semicircle along the outer edge of the Trp-62 indole ring. Thus, zwitterionic ligands such as w-amino acids interact with the anionic and cationic sites at each end of the depression while the aliphatic backbone of each ligand is tubulized by its interaction with the hydrophobic trough. Modeling of the lysine-binding pocket in the mutant and wild-type kringle clearly showed that the Arg72 substitution in the mutant kringle prevented the docking of lysine in the binding pocket.

O. SUBSTITUTION OF Thr66 BY Met66

This mutation is not associated with a lysine-binding and is present in about 45% of the subjects. Modeling of the wild-type and mutant kringle showed that these amino acids are at the kringle surface, remote from the lysine-binding site and thus should not affect lysine binding, in keeping with the results of the clinical studies.

EXAMPLE 8

ASSEMBLY AND SECRETION OF apo(A) AND Lp(A)

The inventors have successfully transfected both HepG2 and McA cells with apo(a) expression vectors encoding an apo(a) with 6, 10 or 17 repeats of the kringle 4 like domain. Each of these constructs also encodes kringle 5 and the protease domain. Single clones have been selected and apo(a) secretion determined quantitatively by ELISA. The highest producing clone was found with the 6 kringle 4 constructs for both rat and human hepatoma cells, with secretion rates from HepG2 cells of 281±57 ng/mg protein/h for total apo(a) of which 77±23 ng/mg protein/h, or about 20% was found as Lp(a). These proportions were confirmed by Western blot analysis of the media from these cells.

Pulse-chase studies using transfected HepG2 cells demonstrated the expected precursor-product relationship for apo(a) and reveal the presence of a B-(a) complex in the media by 60 minutes of chase. Most of the apo(a) in the media at this early time point, however, is uncomplexed (greater than 96% free). The proportions of complexed apo(a) appeared to increase with duration of the chase, reaching about 10% by 5 h.

Transfected HepG2 cells were incubated in serum free medium and washed extensively with heparin, to displace surface-bound LDL. The cells were then lysed in the presence of EACA and intracellular contents immunoprecipitated with an anti apo(a) antiserum, Western blotted with or without reduction and probed with an anti apo B monoclonal antibody. The data clearly showed a B-(a) complex which reduces with DTT. These studies have been repeated using cell surface biotinylation to label the cell surface bound proteins. No biotinylated B-(a) could be detected in the presence of heparin treatment, suggesting that the material identified above was from an intracellular location.

The role of glycosylation of apo(a) upon its ability to form a B-(a) complex has been investigated. In order to examine the effects of alterations in N-linked glycosylation on the secretion of apo(a) from hepatoma cells and whether these alterations influenced the association of apo(a) and apoB100, stably transfected McA7777 cells and HepG2 cells were radiolabeled in the presence of increasing amounts of tunicamycin. Analysis of apo(a) immunoprecipitated from the media of these cells reveals a dose-dependent increase in mobility, evident in both McA7777 and HepG2 cells, consistent with the expected decreases in N-linked sugar addition. Additionally, the small amounts of complexed apo(a)-B100 recovered in the media of untreated HepG2 cells and cells incubated with 2 μg/ml of tunicamycin were undetectable in the media of cells treated with 5 or 10 μg/ml tunicamycin even at longer exposures. It is recognized that tunicamycin treatment may produce effects other than those on the glycosylation of apo(a). Accordingly, a second, independent approach was taken to the question of whether glycosylation alters the ability of apo(a) to associate wit apoB100. Stably transfected McA7777 cells were radiolabeled by pulse-chase and apo(a) immunoprecipitated from lysates prepared at the indicated times.

apo(a) is synthesized as a precursor which undergoes extensive posttranslational processing, resulting in the mature form of the protein which can be identified beginning at 60 minutes of chase. Treatment with brefeldin-A completely blocked the processing

of apo(a) into the mature form, consistent with its previously demonstrated effects in disrupting normal endoplasmic reticulum to Golgi transport of secretory proteins. In order to demonstrate the ability of the precursor form of apo(a) to associate with apoB100, cell lysates were prepared from either control or brefeldin-A treated cells and aliquots incubated with human LDL, followed by immunoprecipitation of apo(a). Lysates prepared from brefeldin-A treated cells were found to support the assembly of a B-(a) complex, indicating that the immature form of apo(a) is competent to associate with apoB100.

To determine whether N- and/or O-linked glycosylation of apo(a) exerts a regulatory role in its eventual association with apoB100, the apo(a) expression plasmid was transfected into several different CHO cell lines with well characterized defects in glycosylation. The apo(a) expression plasmid was transfected into the parental (Pro 5) and four defective CHO cell lines, which were subsequently radiolabeled. The radiolabeled media was mixed with LDL apoB100, followed by immunoprecipitation with anti-apo(a) antisera. All lines secreted apo(a). However, apo(a) secreted from the mutant CHO cells demonstrated altered electrophoretic mobility, as predicted from the differing degrees of glycosylation defect. Mixing studies revealed that apo(a) secreted from all the mutant cells was competent to associate with LDL apoB100. As determined by laser scanning densitometry, the complexed form of apo(a) accounted for approximately 36% of the total apo(a) in the media of Pro5 cells. This value compares with 40% for the Lec1 cells, 39% for Lec2 and 38% for Lec8 cells. The values for *ldlD* cells were somewhat lower at 17%.

The same mixing studies performed with apo(a) secreted from McA7777 cells yielded values for the complexed form of apo(a) of 33 and 40% in duplicate studies. These data provide further evidence that alterations in the glycosylation of apo(a) fail to modulate its association with apoB100. Further studies were performed with the *ldlD* cell line in order to examine separately the question of N- versus O-linked glycosylation. The addition of galactose to these cells permits normal N- and lipid linked

glycosylation but no O-linked sugars are added. In contrast, the addition of N-acetylgalactosamine permitted the synthesis of truncated O-linked chains but without their complement of galactose and sialic acid residues, while addition of both substrates effectively reverses the defects in glycosylation. Stably transfected *ldlD* cells were radiolabeled in the presence of the indicated substrate and aliquots of the media mixed with LDL apoB100. Unsupplemented *ldlD* cells were found to have 18% of the apo(a) in complex form with apoB100. Supplementation with either galactose or N-acetylgalactosamine alone yielded values of 28% and 35%, respectively, while combined supplementation produced a value of 24% apo(a)-B100. These results indicated that correction of the glycosylation defect in the mutant CHO cells produced only modest effects upon the ability of apo(a) to associate with apoB100.

EXAMPLE 9

Lp(a) FUNCTIONAL HETEROGENEITY

Subjects were identified in whom Lp(a) has a defective in lysine and fibrin binding and is present in the plasma in very low levels (< 1.0 mg/ml). In these subjects the DNA isolated from peripheral blood and amplified by PCR™ demonstrated a Trp72→Arg mutation in kringle IV-10. In about 40% of the subjects studied, it was found that the Met66 of kringle IV-10 was replaced by Thr. In the absence of the Trp72→ Arg mutation, the Thr→ Met substitution caused no significant changes in the binding of Lp(a) to lysine/fibrin.

Human wild-type and mutant (Trp72→ Arg) kringle IV-10 has been expressed in both *E. coli* (non-glycosylated form) and CHO cells (glycosylated form). The Arg72 mutant was prepared by introducing the T→ A mutation on apo(a) kringle IV-10 amplified from human liver mRNA by the reverse transcriptase PCR™ technique. The yield of kringles from *E. coli* was significantly higher than that from CHO cells. The Met66→ Thr mutant has also been expressed on *E. coli*.

The non-glycosylated wild-type apo(a) kringle IV-10 expressed in *E. coli* bound readily to lysine Sepharose™; the binding was inhibited by ε-aminocaproic acid (EACA) indicating lysine dependency. The kringle bound bimodally to plasmin-modified fibrinogen (PM-fibrinogen): one component was lysine sensitive (inhibitable by EACA), the other was lysine insensitive. The two components occurred in about the same proportion. the glycosylated wild-type kringle IV-10 expressed in CHO cells also bound to lysine and PM-fibrinogen. On the other hand, the mutant Trp72→ Arg mutant failed to bind to lysine and bound to PM-fibrinogen only through its non-lysine mediated component. Overall, these results have provided further support to the notion that kringle IV-10 and in particular Trp72 plays a dominant role in lysine/fibrin binding and that this binding is not significantly affected by glycosylation.

Starting from genomic DNA extracted from the cells of peripheral blood, the introns between the regions of kringle V and protease domain were amplified. The amplification primers were selected by assuming that the position of the three introns was the same as in the plasminogen gene.

Three sets of primers were used:

#1 5′-TGTCATCCAGGTTCCAAG-3 (SEQ ID NO:1)

5′-TTATTTGTCCCTGGAATGAACG-3′ (SEQ ID NO:2)

#2 5′-AGCCCCATAGACACAGCACG-3′ (SEQ ID NO:3)

5′-AGCACCAGGGACCATTGATG-3′ (SEQ ID NO:4)

#3 5′-TAACCCTGATGGTGACATCAA-3′ (SEQ ID NO:5)

5′-CACACACCCCCCTACAATG-3′ (SEQ ID NO:6)

The primer set #1 amplified a 700 bp length fragment encompassing the area of kringle IV-37 exon b, intron 1 and part of the KV exon a. The primer set #2 amplified a 2.2 kb fragment encompassing part of KV exon a, intron 2 and part of the kringle V exon b. Primer set #3 amplified a 2.9 kb fragment encompassing part of the kringle V exon b, intron 3 and a part of the protease region exon. Each fragment was amplified by PCR™ by carefully selected conditions, then gel purified using a QIAEX® kit (Qiagen) and directly cloned into a TA vector (Invitrogen) using the "original" TA cloning kit. The positive clones (with inserts) were identified by blue/white color selection, digested with *Eco*R1 and the digests sequenced by using the "fmol® DNA sequence system" (Promega) using as sequencing primers the same ones employed for PCR™ amplification. The sequences obtained for the kringle V and the protease regions corresponded to those previously reported from cDNA sequencing. The intronic regions were homologous but not identical to those on the plasminogen gene. The knowledge of the sequence of three introns allowed the generation of primers for the amplification of the exons coding for the KV and protease domains.

EXAMPLE 10

LIPOPROTEIN HETEROGENEITY STRUCTURE AND FUNCTION

A. INTERACTION OF Lp(a) WITH CELLS

This study was predicated on observations showing that Lp(a) competes with plasminogen for binding to cells. Nevertheless, direct binding of intact Lp(a) particles to plasminogen receptors had not been directly demonstrated. Moreover, other studies have focused on LDL, scavenger, and/or unique apo(a) receptors as the primary binding mechanisms of Lp(a). Direct binding of Lp(a) to plasminogen binding sites has been examined on human monocytoid U937 cells and umbilical vein endothelial cells. Based upon the capacity of plasminogen and lysine analog to inhibit ^{125}I-binding, an

interaction of Lp(a) with the plasminogen receptors on these cells was demonstrable. The interaction of Lp(a) with these sites was time-dependent, specific, saturable divalent ion independent and temperature-sensitive, characteristics of plasminogen binding to these same sites. The affinity of plasminogen and Lp(a) for these sites also was similar (K_d equal to 1-3 μM), but Lp(a) bound to fewer sites (10-fold less). This difference in the number of binding sites may reflect steric limitations in Lp(a) binding or the recognition of subpopulations of plasminogen binding sites. Cell surface proteins with carboxy-terminal lysyl residues, including a-enolase, a candidate plasminogen receptor, inhibited Lp(a) binding to U937 cells. An additional and previously undescribed interaction of Lp(a) with cells involving a low affinity interaction with non-selective (recognizing both LDL and HDL) lipoprotein binding sites was also demonstrated. Binding of Lp(a) to these sites, as well as to plasminogen receptors, was inhibited by gangliosides but not the constituents of gangliosides. Thus, Lp(a) can interact with plasminogen binding sites on cells *via* its LBS, providing support for the hypothesis.

B. INTERACTION OF Lp(a) WITH THE EXTRACELLULAR MATRIX

Studies have progressed to examine the interaction of Lp(a) with the extracellular matrix. For these studies, Matrigel (the extracellular matrix of murine tumor cells) was used. When immobilized on microtiter plates, this matrix bound by ^{125}I-Lp(a) in a time-dependent manner, and the extent of Lp(a) binding was dependent upon the amount of matrix provided. This binding was specific and saturable; it was not inhibited by unrelated proteins, but was inhibited a nonlabeled Lp(a). ^{125}I-plasminogen binding to the matrix also was demonstrable. Interaction of both ligands was inhibited EACA, implicating their LBS in binding. However, Lp(a), but not plasminogen binding, also was inhibited by selected hydrophobic amino acids, suggesting an additional contribution to Lp(a) binding. Most notably, under conditions where substantial Lp(a) binding to the matrix could be demonstrated, LDL did not bind. Only at higher concentrations of LDL was an interaction demonstrable. These data strongly

supported the concept of a unique interaction of Lp(a), as contrasted to LDL, with the extracellular matrix.

C. QUANTITATIVE IMMUNOASSAY FOR LBS-Lp(a)

In view of the importance of the LBS for the interaction of Lp(a) with cells and matrices and the reported heterogeneity of the LBS function of Lp(a) in different individuals, a quantitative immunoassay has been developed to measure the LBS function of Lp(a) in plasma. Intrinsic advantages to such an assay include: 1) less cumbersome than lysine-Sepharose™ chromatography; 2) adaptable to multiple plasma samples; 3) quantitative rather than qualitative results; *i.e.*, distinguish the capacity to affinity differences of the LBS as opposed to simple retention on lysine-Sepharose™); and 4) applicable to small plasma sample volumes. Such an assay has been successfully developed.

An antibody was identified that reacts with Lp(a) in the absence of EACA, but not its presence. This anti-LBS also did not react with Lp(a) or apo(a) with impaired LBS function (natural or induced mutations). In one embodiment, Lp(a) is captured from plasma with a monoclonal antibody specific for apo(a). The immunocaptured Lp(a) is then reacted with anti-LBS. Binding of the anti-LBS antibody is quantitated with an alkaline phosphatase conjugated disclosing antibody. Using this assay, the LBS activity of an unknown Lp(a) sample can be quantitated relative to a reference Lp(a) standard (fully retained on lysine-Sepharose™). LBS-Lp(a) values in plasma samples ranged from 0 - 100% of total Lp(a) levels. Notably, the LBS-Lp(a) activity correlated poorly with total Lp(a) concentrations in the plasma. Thus, this assay established marked heterogeneity in the LBS function of Lp(a) from different individuals and may distinguish pathogenetic from non-pathogenic forms of Lp(a). The LBS-Lp(a) assay also has been used to assess whether metabolic modification alters LBS function. Lp(a) was preincubated with lipoprotein lipase, sphingomyelinase, phospholipase C, phospholipase A2 or thrombin was assessed for alterations of LBS activity. The most

marked change in LBS activity was induced by phospholipase A2, which resulted in a 56% increase in the LBS activity.

D. TRANSPORT OF Lp(a) INTO A SUBENDOTHELIAL MATRIX

A two-compartment system, with a monolayer of bovine aortic endothelial cells forming a barrier, was used to compare the transport and retention of Lp(a) and LDL into the subendothelial matrix. Baseline values for transport and retention of Lp(a) and LDL were not significantly different. Incubation with sphingomyelinase or lipoprotein lipase caused modest and similar increases (1.2-2.0-fold, respectively) in transport and retention of the two lipoproteins. In contrast, incubation with phospholipase A2 (PLA2), caused a 4-fold increase in retention of Lp(a) on the subendothelial matrix, but only a 2-fold increase in LDL retention. PLA2 did not have a differential effect on the binding of Lp(a) and LDL to the cells, suggesting that PLA2 increased retention of Lp(a) in the subendothelial matrix. When PLA 2 was present, retention of Lp(a) on membranes (without cells) coated with extracellular proteins (fibronectin, laminin, or collagen 1) was 4-10 times higher than LDL. Thus, the increase in binding to the subendothelial matrix may be related to an enhanced LBS function, arising from the modification of Lp(a) by PLA2.

* * * * * * * * * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described

herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following literature citations as well as those cited above are incorporated in pertinent part by reference herein for the reasons cited in the above text:

Albers and Marcovina, "Lipoprotein(a) quantification: comparison of methods and strategies for standardization," *Curr. Opin. Lipidology*, 5:417-421, 1994.

Bartlett, *J. Biol. Chem. 234*, 466-468, 1959.

Beisiegel, "Lipoprotein(a) in the Arterial Wall," *Curr. Opin. Lipidology*, 2:317-323, 1991.

Bersot *et al.*, "Fat Feeding in Humans Induces Lipoproteins of Density Less Than 1.006 that are Enriched in apolipoprotein(a) and that Cause Lipid Accumulation in Macrophages," *J. Clin. Invest.*, 77:622-630, 1986.

Bode *et al.*, *Biochemistry*, 28:1951-1963, 1989

Brunner *et al.*, "Cys(4057) of apolipoprotein(a) is Essential for Lipoprotein(a) assembly," *Proc. Nat'l. Acad. of Sci. USA*, 90:11643-11647, 1993.

Callow *et al.*, *Proc. Nat'l. Acad. Sci. U.S.A .91*, 2130-2134, 1994.

Carrell *et al.*, *Nature*, 298:239-334, 1982

Castell et al., "Presence of Fibronectin Fragments in Bronchalveolar Lavages" Biochem. Soc. Trans. 16: 378-379, 1988

Chiesa *et al.*, "Reconstitution of Lipoprotein(a) by Infusion of Human Low Density Lipoprotein into Transgenic Mice Expressing Human apolipoprotein(a)," *J. Biol Chem.*, 267: 24369-24374, 1992.

Chung and Scanu, "Isolation, Molecular Properties, and Kinetic Characterization of Lipoprotein Lipase from Rat Heart," *J. Biol. Chem.*, 252:4202-4209, 1977.

Curtiss *et al.*, "Interaction of Lp(a) with Plasminogen Binding Sites on Cells," *Thromb. Haemost.*, 73:458-465, 1995.

Edelstein and Scanu, *Methods Enzymol. 128,* 151-155, 1986.

Edelstein *et al.*, "Hypoalphalipoproteinemia: Postprandial Response of Subjects with Preprandial Normal and Hypertriglyceridemia to Various Diets," *Metabolism,* 42:1-8, 1993.

Edelstein *et al.*, "Oleate Stimulates the Formation of Triglyceride-Rich Particles Containing apoB100-apo(a) in Long-Term Primary Cultures of Human Hepatocytes," *Chem. Phys. Lipids*, 67-8:135-143, 1994.

Edelstein *et al.*, "Determinants of Lipoprotein (a) assembly: A Study of Wild-type and Mutant Apolipoprotein Phenotypes Isolated from Human and Rhesus Monkey, Lipoprotein (a) Under Mild Reductive Conditions" *Biochemistry*, 34: 16483-16493, 1995.

Ernst *et al.*, "Identification of Two Functionally Distinct Lysine-Binding Sites in Kringle 37 and in Kringles 32-36 of Human apolipoprotein(a)," *J. Biol. Chem.*, 270: 6227-6234, 1995.

Fless *et al.*, "Physicochemical Properties of apolipoprotein(a) and Lipoprotein(a-) Derived from the Dissociation of Human Plasma Lipoprotein (a)," *J. Biol. Chem.*, 1986:8712-8718, 1986.

Fless *et al.*, "Polymorphic Forms of Lp(a) with Different Structural and Functional Properties: Cold-Induced Self-Association and Binding to Fibrin and Lysine-Sepharose," *Chem. Phys. Lipids*, 67/68:69-79, 1994.

Fless *et al.*, "Solubility, Immunochemical, and Lipoprotein Binding Properties of apoB-100-apo(a), the Protein Moiety of Lipoprotein(a)," *J. Lipid Res.*, 31:909-918, 1990.

Fless *et al.*, "Subunit Composition of Lipoprotein(a) Protein," *Biochemistry*, 33:13492-13501, 1994.

Fless *et al.*, *J. Lipid Res.*, 30:651-662, 1989.

Fleury and Angles-Cano, "Characterization of the Binding of Plasminogen to Fibrin Surfaces: The Role of Carboxy-terminal Lysines," *Biochemistry*, 30:7630-7638, 1991.

Frank *et al.*, "Structural Requirements of apo-a for the Lipoprotein-a Assembly," *Biochem. J.*, 304: 27-30, 1994a.

Frank *et al.*, "High-level Expression of Various apolipoprotein(a) Isoforms by "Transferrinfection": The Role of Kringle IV Sequences in the Extracellular Association with Low-Density Lipoprotein," *Biochemistry*, 33:12329-12339, 1994b.

Gabel *et al.*, "Carboxyl-terminal Truncation of apolipoproteinB-100 Inhibits Lipoprotein(a) Particle Formation," *FEBS Letters*, 350:77-81, 1994.

Gianturco and Bradley, "The Role of apolipoprotein Processing in Receptor Recognition of VLDL," *Meth. Enz.*, 128:319-344, 1986.

Grainger *et al.*, "Proliferation of Human Smooth Muscle Cells Promoted by Lipoprotein(a)," *Science*, 260:1655-1658, 1993.

Guevara *et al.*, *Arteriosclerosis Thromb.*, 13: 758-770, 1993.

Guevara *et al.*, *Proteins - Struct. Funct. Genet.*, 12:188-199, 1992.

Harpel *et al.*, "Plasmin Catalyzes Binding of Lipoprotein(a) to Immobilized Fibrinogen and Fibrin," *Proc. Nat'l. Acad. Sci. USA*, 86:3847-3851, 1989.

Havel and Kane, "Structure and metabolism of plasma lipoproteins" In: *The Metabolic Basis of Inherited Disease, 6th edn.* Scriver et al., Eds. McGraw-Hill publ. Vol. II: 1129-1138, 1992.

Hoover-Plow *et al.*, "A Quantitative Immunoassay for the Lysine Binding Function of Lipoprotein(a) in Plasma," *Fibrinolysis*, 8:349a, 1994.

Hoover-Plow *et al.*, "Comparison of the Lysine Binding Functions of Lipoprotein(a) and Plasminogen," *Biochemistry*, 32:13681-13687, 1993.

Hoover-Plow *et al.*, "Phospholipase A2 Preferentially Increases Lp(a) Binding to the Subendothelial Matrix," *FASEB J.*, 9:A766, 1995.

Huby *et al.*, "Characterization of the N-terminal and C-terminal Domains of Human apolipoprotein(a): Relevance to Fibrin Binding," *Biochemistry*, 34:7385-7393, 1995.

Huby *et al.*, "Structural Domains of apolipoprotein(a) and Its Interaction with apolipoprotein B-100 in the Lipoprotein(a) Particle," *Biochemistry*, 33:3335-3341, 1994.

Iverius and Lindqvist-Ostlund, "Preparation, Characterization, and Measurement of Lipoprotein Lipase," *Meth. Enzymol.*, 128:691-704, 1986.

Klezovitch and Scanu, "Heterogeneity of Lipoprotein(a): Growing Complexities," *Curr. Opin. Lipidology*, 6:223-228, 1995.

Klezovitch and Scanu, "Lysine and Fibrogen Binding of Wild-type (Trp72) and Mutant (Arg72) Human apolipoprotein(a) Kringle IV-10 Expressed in E. coli and CHO Cells," *Arterioscl. Thromb. Vascul. Biol.*, 1996.

Koschinsky *et al.*, *Biochemistry*, 30:5044-5051, 1991.

Koschinsky *et al.*, *J. Biol. Chem.*, 268:19819-19825, 1993.

Krauss, "Dense Low Density Lipoproteins and Coronary Artery Disease," *Am. J. Card.*, 75:B53-B57, 1995.

Kraunsoe *et al.*, "Inhibition of Human Leukocyte and Porcine Pancreatic Elastase by Homologues of Bovine Pancreatic Trypsin Inhibitor," *Biochemistry*, 35:9090-9096, 1996

Kreuzer *et al.*, "Lipoprotein(a) Displays Increased Accumulation Compared with Low-Density Lipoprotein in the Murine Arterial Wall," *Chem. Phys. Lipids*, 67/68:175-190, 1994.

Lackner *et al.*, *J. Clin. Invest.*, 87:2153-2161, 1991.

Lawn *et al.*, "Atherogenesis in Transgenic Mice Expressing Human apolipoprotein(a)," *Nature*, 360:670-672, 1992.

Levin *et al.*, "Lipoproteins Inhibit the Secretion of Tissue Plasminogen Activator from Human Endothelial Cells," *Arterioscler. Thromb.*, 14:438-442, 1994.

Li *et al.*, *Protein Express. Purif.*, 3:212-222, 1992.

Lindgren *et al.*, "In Blood Lipids and Lipoproteins," G.J. Nelson, Editor, Wiley (Interscience): New York, p. 181, 1972.

Linton *et al.*, *J. Clin. Invest.*, 92:3029-3037, 1993.

LoGrasso *et al.*, *J. Biol. Chem.*, 269:21820-21827, 1994.

Loscalzo *et al.*, "Lipoprotein(a), Fibrin Binding, and Plasminogen Activation," *Arteriosclerosis*, 10:240-245, 1990.

Makino *et al.*, "Effect of Glycationon the Properties of Lp(a)," *Arterioscler. Thromb. Vasc. Biol.*, 15:385-391, 1995.

Manavalan and Johnson, "Variable Selection Method Improves the Prediction of Protein Secondary Structure from Circular Dichroism Spectra," *Analyt. Biochem.*, 167:76-85, 1987.

McCormick *et al.*, "Expression of Human apolipoprotein b90 in Transgenic Mice - Demonstration that apolipoprotein b90 Laces the Structural Requirements to form Lipoprotein(a)," *J. Biol. Chem.*, 269:24284-24289, 1994.

McLean *et al.*, "cDNA Sequence of Human apolipoprotein(a) is Homologous to Plasminogen," *Nature*, 330:132-137, 1987.

Miles *et al.*, "Interaction of Lipoprotein(a) [Lp(a)] with the Extracellular Matrix," *Blood*, 82:(Suppl.)175a, 1993.

Miles *et al.*, "Interaction of Lp(a) with Plasminogen Binding Sites on Cells," *Thromb. Haemost.*, 73:458-465, March 1995.

Morrisett *et al.*, "Metabolism of apo(a) and apoB100 in Human Lipoprotein(a), in Drugs Affecting Lipid Metabolism," A.L. Catapano, Gotto, A.M. Jr., Smith, L.C., Editor, Kluwer: Boston, p. 161, 1993.

Nichols *et al.*, "Nondenaturing Polyacrylamide Gradient Gel Electrophoresis," *Methods Enz.*, 128:417-431, 1986.

Nilsson *et al.*, *Anal. Biochem.*, 110:342-348, 1981.

Phillips *et al.*, *Biochemistry*, 32:3722-3728, 1993.

Provencher and Glockner, "Estimation of Globular Protein Secondary Structure from Circular Dichroism," *Biochemistry*, 20:33-37, 1981.

Rader *et al.*, "The Inverse Association of Plasma Lipoprotein(a) Concentrations with apolipoprotein(a) Isoform Size is not Due to Differences in Lp(a) Catabolism but to Differences in Production Rate," *J. Clin. Invest.*, 93:2758-2763, 1994.

Rath *et al.*, "Detection and Quantification of Lipoprotein(a) in the Arterial Wall of 107 Coronary Bypass Patients," *Arteriosclerosis*, 9:579-592, 1989.

Scanu, "Identification of Mutations in Human apolipoprotein(a) Kringle 4-37 from the Study of the DNA of Peripheral Blood Lymphocytes: Relevance to the Role of Lipoprotein(a) in Atherothrombosis," *Amer. J. Cardiol.*, 75:58B-61B, 1995.

Scanu, "Structural and Functional Polymorphism of Lipoprotein(a): Biological and Clinical Implications," *Clin. Chem.*, 41:170-172, 1995.

Scanu, "Structural Basis for the Presumptive Atherothrombogenic Action of Lipoprotein(a)-Facts and Speculations," *Biochem. Pharm.*, 46:1675-1680, 1993.

Scanu and Edelstein, "apolipoprotein(a): Structural and Functional Consequences of Mutations in Kringle Type 10 (or Kringle 4-37)," *Clin. Genet.*, 46:42-45, 1994.

Scanu and Edelstein, "Kringle-Dependent Structural and Functional Polymorphism of apolipoprotein(a)," *Biochim. Biophys. Acta*, 1256:1-12, 1995.

Scanu and Fless, *Clin.Invest.*, 85:1709-1715, 1990.

Scanu *et al.*, "A Single Point Mutation (Trp72 → Arg) in Human apo(a) Kringle 4-37 Associated with a Lysine Binding Defect in Lp(a)," *Biochim. Biophys. Acta*, 1227:41-45, 1994.

Scanu *et al.*, "Genetically-Determined Polymorphism of Kringle 4-37 of Human apolipoprotein(a)," *Circulation*, 90:3357, 1994.

Scanu *et al.*, "Lipoprotein(a) and Atherosclerosis," *Ann. Int. Med.*, 115:209-218, 1991.

Scanu *et al.*, "Postprandial Lipoprotein(a) Response to a Single Meal Containing Either Saturated or Omega-3 Polyunsaturated Fatty Acids in Subjects with Hypoalphalipoproteinemia," *Metabolism*, 41:1361-1366, 1992.

Scanu *et al.*, "Postprandial Lp(a): Identification of Triglyceride-Rich Particle Containing apoE," *Chemistry and Physics of Lipids*, 67/68:193-198, 1994.

Scanu *et al.*, *J. Clin. Invest.*, 91:283-291, 1993.

Scanu, *J. Lab. Clin. Med.*, 116:142-146, 1990.

Schumaker and Puppione, *Methods Enzymol. 128*, 155-170, 1986.

Selinger *et al.*, *Clin. Biochem. 26*, 59-64, 1993.

Snyder *et al.*, "Binding and Degradation of Lipoprotein(a) and LDL by Primary Cultures of Human Hepatocytes: Comparison with Cultured Human Monocyte-Macrophages and Fibroblasts," *Arterioscler. Thromb.*, 14:770-779, 1994.

Snyder *et al.*, "Comparative Binding and Degradation of Lipoprotein(a) and Low Density Lipoprotein by Human Monocyte-Derived Macrophages," *J. Biol. Chem.*, 267:339-346, 1992.

Sottrup-Jensen *et al.*, eds, "The Primary Structure of Human Plasminogen: Isolation of Two Lysine Binding Fragments and One 'Mini' -Plasminogen (MW 38,000) by Elastase Catalyzed Specific Limited Proteolysis," *Prog. Chem Fibrinolysis Thrombolysis*, ed. R.R. Davidson JF, Samana MM, Desnoyes PC., Raven Press: New York. 191-209, 1978.

Steyrer *et al.*, "The Role of Lecithin: Cholesterol Acyltransferase for Lipoprotein(a) Assembly -Structural Integrity of Low Density Lipoproteins is a Prerequisite for Lp(a) Formation in Human Plasma," *J. Clin. Invest.*, 94:2330-2340, 1994.

Tetley, T.D. *Thorax*, 48: 560-565, 1993

Tomlinson *et al.*, "Rhesus Monkey apolipoprotein(a)," *J. Biol. Chem.*, 264:5957-5965, 1989.

Trieu and McConathy, "A Two-Step Model for Lipoprotein(a) Formation," *J. Biol. Chem.*, 270:15471-15474, 1995.

Trieu *et al.*, "The apolipoprotein B3304-3317 Peptide as an Inhibitor of the Lipoprotein (a):apolipoprotein B-Containing Lipoprotein Interaction," *Biochem. J.*, 307:17-22, 1995.

Trieu *et al.*, *J. Biol. Chem.*, 266:5480-5485, 1991.

Utermann, "Lipoprotein(a). The Metabolic and Molecular Basis of Inherited Disease," A.L.B. C.R. Scriver, W.S. Sly and D. Valle, ed., McGraw Hill, Inc., New York, pgs. 1887-1912, 1995.

Van Der Hoek *et al.*, "Analysis of Structure-Function Relationships in Human apolipoprotein(a)," *Canad. J. Phys. and Pharm.*, 72:304-310, 1994.

White and Lanford, "Cell Surface Assembly of Lipoprotein(a) in Primary Cultures of Baboon Hepatocytes," *J. Biol. Chem.*, 269:28716-28723, 1994.

White *et al.*, *J. Lipid Res. 34*, 509-517, 1993.

Wilkie and Landry, *BioChromat. 5*, 205-215, 1988.

Williams *et al.*, "Mechanism by which Lipoprotein Lipase Alters Cellular Metabolism of Lipoprotein(a), Low density lipoprotein, and nascent lipoproteins. Roles for low density Lipoprotein Receptors and Heparan Sulfate Proteoglycans," *J. Biol. Chem.*, 267:13284-13292, 1992.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: ARCH DEVELOPMENT CORPORATION
(B) STREET: 1101 EAST 58TH STREET
(C) CITY: CHICAGO
(D) STATE: IL
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 60637
(G) TELEPHONE: 512/418-3000
(H) TELEFAX: 512/713-789-2679

(ii) TITLE OF INVENTION: ISOLATION OF apo(a), COMPOSITIONS, AND METHODS OF USE

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/006,395
(B) FILING DATE: 09-NOV-1995

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/691,795
(B) FILING DATE: 02-AUG-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGTCATCCAG GTTCCAAG 18

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTATTTGTCC CTGGAATGAA CG 22

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGCCCCATAG ACACAGCACG 20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGCACCAGGG ACCATTGATG 20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TAACCCTGAT GGTGACATCA A 21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CACACACCCC CCTACAATG 19

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Glu Gln Ser His Val Val Gln Asp Cys Tyr His Gly Asp Gly Gln Ser
1 5 10 15

Tyr Arg Gly Thr
20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:14
(D) OTHER INFORMATION:/product= "OTHER"
/note= "X = Unknown/Undetermined"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Glu Gln Ser His Val Val Gln Asp Cys Tyr His Gly Asp Xaa Gln Ser
1 5 10 15

Tyr Arg Gly Thr
20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:one-of(4, 14)
(D) OTHER INFORMATION:/product= "OTHER"
/note= "X = Unknown/Undetermined"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:16..17
(D) OTHER INFORMATION:/product= "OTHER"
/note= "X = Unknown/Undetermined"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Leu Ala Pro Xaa Leu Glu Ala Phe Phe Glu Gln Ala Leu Xaa Glu Xaa
1 5 10 15

Xaa Pro

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Thr Ala Phe Val Pro Pro Asn Val Ile Leu Ala Pro Ser Leu Glu Ala
1 5 10 15

Phe Phe Glu Gln Ala Leu Thr Glu Glu Thr Pro
20 25

CLAIMS:

1. A method for the purification of apolipoprotein (a) comprising the steps of:

a) providing a composition comprising lipoprotein (a);

b) contacting said lipoprotein (a) composition with a reducing agent;

c) further contacting said lipoprotein (a) with a lysine analog to produce a reaction mixture containing lipoprotein (a), reducing agent, and lysine analog;

d) incubating said mixture under conditions whereby LDL, unreacted lipoprotein (a) and free apolipoprotein (a) are produced; and

e) separating said apolipoprotein (a) from said mixture.

2. The method of claim 1, wherein the reducing agent is selected from the group consisting of homocysteine, N-acetyl cysteine, 2-mecaptoethanol, 3-mercaptopropionate, 2-aminoethanol, dithiothreitol, and dithioerythritol.

3. The method of claim 2, wherein the reducing agent is dithioerythritol in a concentration between about 0.5mM and 2.0mM.

4. The method of claim 1, wherein the lysine analog is selected from the group consisting of trans 4(amino-methyl)-cyclohexanecarboxylic acid, N-acetyl-L-lysine, p-benzylamine sulfonic acid, hexylamine, benzamidine, benzylamine, L-proline and e-aminocaproic acid.

5. The method of claim 4, wherein the lysine analog is e-aminocaproic acid present in a concentration between 50mM and 200mM.

6. The method of claim 1, wherein said separating of apolipoprotein (a) is achieved by ultracentrifugation.

7. A method for the purification of fragments of apolipoprotein (a) comprising the steps of:

a) providing a composition comprising lipoprotein (a);

b) contacting said lipoprotein (a) composition with a reducing agent;

c) further contacting said lipoprotein (a) composition with a lysine analog to produce a reaction mixture containing lipoprotein (a), reducing agent, and lysine analog;

d) incubating said mixture under conditions whereby LDL, unreacted lipoprotein (a) and free apolipoprotein (a) are produced;

e) separating apolipoprotein (a) from said mixture;

f) contacting said apolipoprotein (a) with a concentration of a proteolytic enzyme whereby fragments of apolipoprotein (a) are produced; and

g) separating said fragments.

8. The method of claim 7, wherein the reducing agent is selected from the group consisting of homocysteine, N-acetyl cysteine, 2-mecaptoethanol, 3-mercaptopropionate, 2-aminoethanol, dithiothreitol, and dithioerythritol.

9. The method of claim 8, wherein the reducing agent is dithioerythritol in a concentration between about 0.5mM and 2.0mM.

10. The method of claim 7, wherein the lysine analog is selected from the group consisting of trans 4(amino-methyl)-cyclohexanecarboxylic acid, N-acetyl-L-lysine, p-benzylamine sulfonic acid, hexylamine, benzamidine, benzylamine, L-proline and e-aminocaproic acid.

11. The method of claim 10, wherein the lysine analog is e-aminocaproic acid present in a concentration between 50mM and 200mM.

12. The method of claim 7, wherein said separating of apolipoprotein (a) is achieved by ultracentrifugation.

13. The method of claim 7, wherein the proteolytic enzyme cleaves the bond between Ile3520-Leu3521 of apolipoprotein (a) to produce an F1 fragment and an F2 fragment.

14. The method of claim 13, wherein the proteolytic enzyme is elastase.

15. The method of claim 13, wherein the F1 fragment produced has an apparent molecular weight of about 220kDa and wherein said molecular weight varies with size of the phenotype.

16. The method of claim 13, wherein the F2 fragment produced has an apparent molecular weight of about 170kDa.

17. A method of screening for elastase activity in diseased tissue comprising the steps of:

a) contacting said tissue with a composition comprising apolipoprotein a;

b) determining the presence of fragments of apolipoprotein (a), wherein the presence of fragments is indicative of the presence of elastase activity in the diseased state.

18. The method of claim 17, wherein the fragments comprise either an F1 fragment, an F2 fragment or a mixture thereof.

19. The method of claim 18, wherein the F1 fragment has an apparent molecular weight of about 220kDa wherein said molecular weight varies with size of the phenotype.

20. The method of claim 18, wherein the F2 fragment has an apparent molecular weight of about 170kDa.

21. A method of screening for inhibitors of elastase activity comprising the steps of:

a) obtaining apolipoprotein (a);

b) contacting said apolipoprotein (a) with elastase and a candidate substance for the inhibition of elastase activity; and

c) comparing the cleavage products of apolipoprotein (a) in the presence of the candidate substance with the cleavage products in the absence of the candidate substance;
whereby the lack of fragments F1 and F2 in the presence of the candidate substance is indicative of inhibition of elastase activity.

22. An apolipoprotein (a) formed according to a method comprising the steps of:

a) providing a composition comprising lipoprotein (a);

b) contacting said lipoprotein (a) composition with a reducing agent;

c) further contacting said lipoprotein (a) composition with a lysine analog to produce a reaction mixture containing lipoprotein (a), reducing agent, and lysine analog;

d) incubating said reaction mixture under conditions whereby LDL, unreacted lipoprotein (a) and free apolipoprotein (a) are produced.

23. The method of claim 22 further comprising separating said apolipoprotein (a) from said mixture.

24. The method of claim 22, wherein the reducing agent is selected from the group consisting of homocysteine, N-acetyl cysteine, 2-mecaptoethanol, 3-mercaptopropionate, 2-aminoethanol, dithiothreitol, and dithioerythritol.

25. The method of claim 24, wherein the reducing agent is dithioerythritol present in a concentration between about 0.5mM and 2.0mM.

26. The method of claim 22, wherein the lysine analog is selected from the consisting of trans 4(amino-methyl)-cyclohexanecarboxylic acid, N-acetyl-L-lysine, p-benzylamine sulfonic acid, hexylamine, benzamidine, benzylamine, L-proline and e-aminocaproic acid.

27. The method of claim 26, wherein the lysine analog is e-aminocaproic acid present in a concentration between 50mM and 200mM.

28. A fragment of apolipoprotein (a) formed according to a method comprising the steps of:

a) providing a composition comprising lipoprotein (a);

b) contacting said lipoprotein (a) composition with a reducing agent;

c) further contacting said lipoprotein (a) composition with a lysine analog to produce a reaction mixture containing lipoprotein (a), reducing agent, and lysine analog;

d) incubating said reaction mixture under conditions whereby LDL, unreacted lipoprotein (a) and free apolipoprotein (a) are produced;

e) separating apolipoprotein (a) from said mixture;

f) contacting said apolipoprotein (a) with a concentration of a proteolytic enzyme to produce fragments of apolipoprotein (a).

29. The method of claim 28, wherein the said fragments are separated into purified fractions.

30. The method of claim 28, wherein the reducing agent is selected from the group consisting of homocysteine, N-acetyl cysteine, 2-mecaptoethanol, 3-mercaptopropionate, 2-aminoethanol, dithiothreitol, and dithioerythritol.

31. The method of claim 30, wherein the reducing agent is dithioerythritol present in a concentration between about 0.5mM and 2.0mM.

32. The method of claim 28, wherein the lysine analog is selected from the consisting of trans 4(amino-methyl)-cyclohexanecarboxylic acid, N-acetyl-L-lysine, p-benzylamine sulfonic acid, hexylamine, benzamidine, benzylamine, L-proline and e-aminocaproic acid.

33. The method of claim 32, wherein the lysine analog is e-aminocaproic acid present in a concentration between 50mM and 200mM.

34. The method of claim 28, wherein the proteolytic enzyme cleaves the bond between Ile3520-Leu3521 of apolipoprotein (a) to produce an F1 fragment and an F2 fragment.

35. The method of claim 34, wherein the proteolytic enzyme is elastase.

36. The method of claim 34, wherein the F1 fragment produced has a molecular weight of about 220kDa.

37. The method of claim 34, wherein the F2 fragment produced has a molecular weight of about 170kDa.

38. An antibody immunologically reactive with purified lipoprotein (a).

39. An antibody immunologically reactive with an F1 fragment of apolipoprotein (a).

40. An antibody immunologically reactive with an 170kDa fragment of apolipoprotein (a).

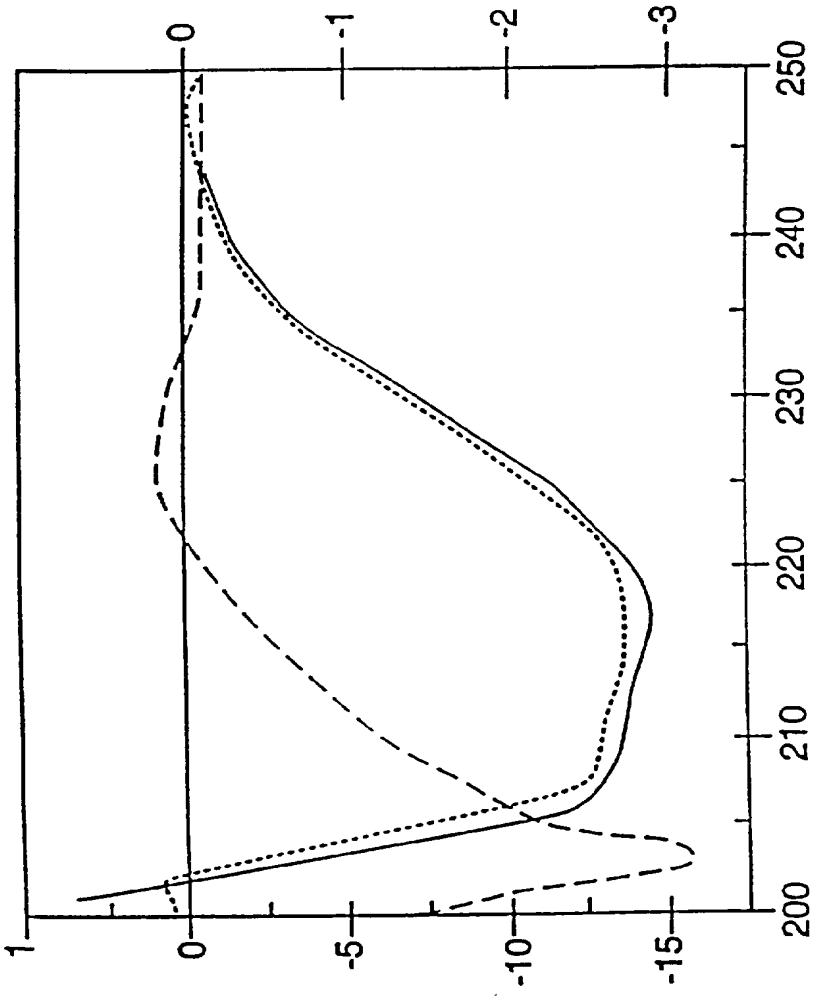

FIG. 1

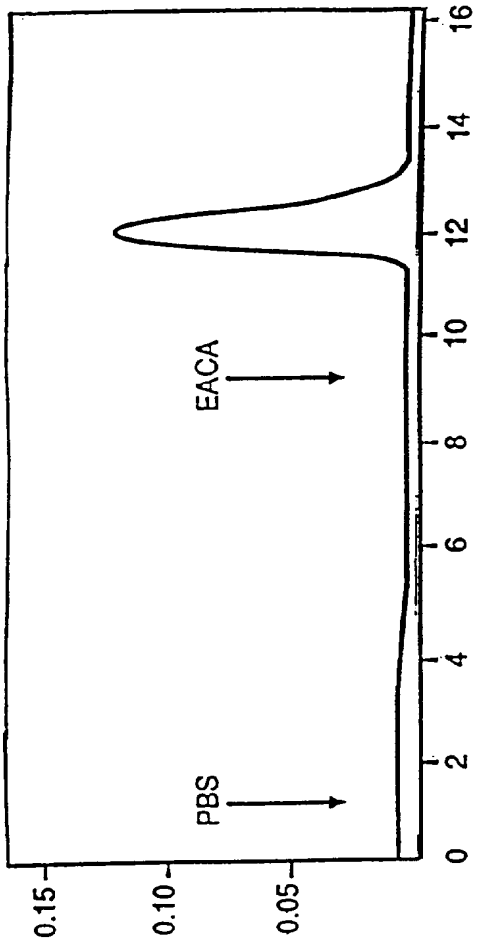

FIG. 2A

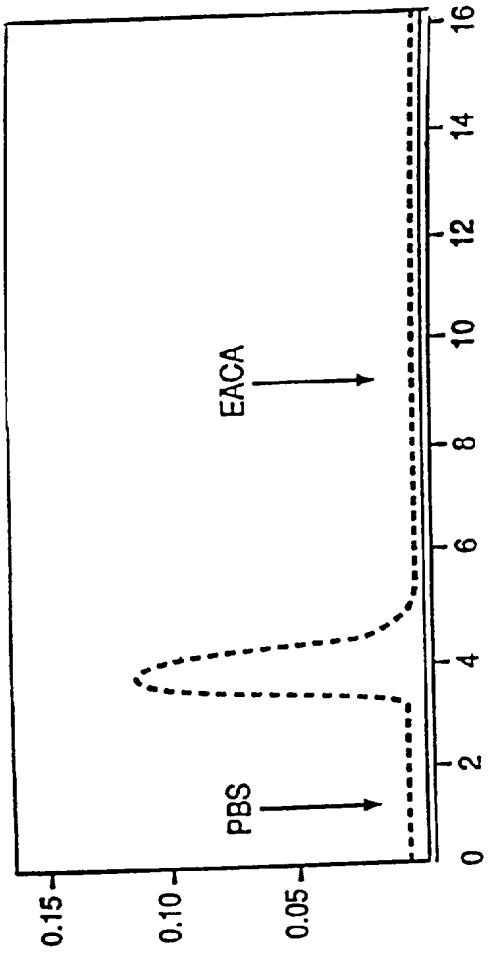

FIG. 2B

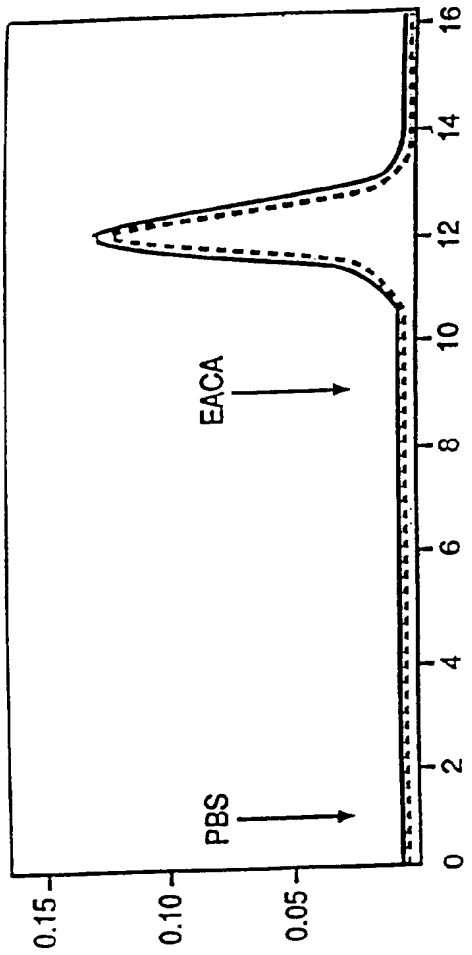

FIG. 2C

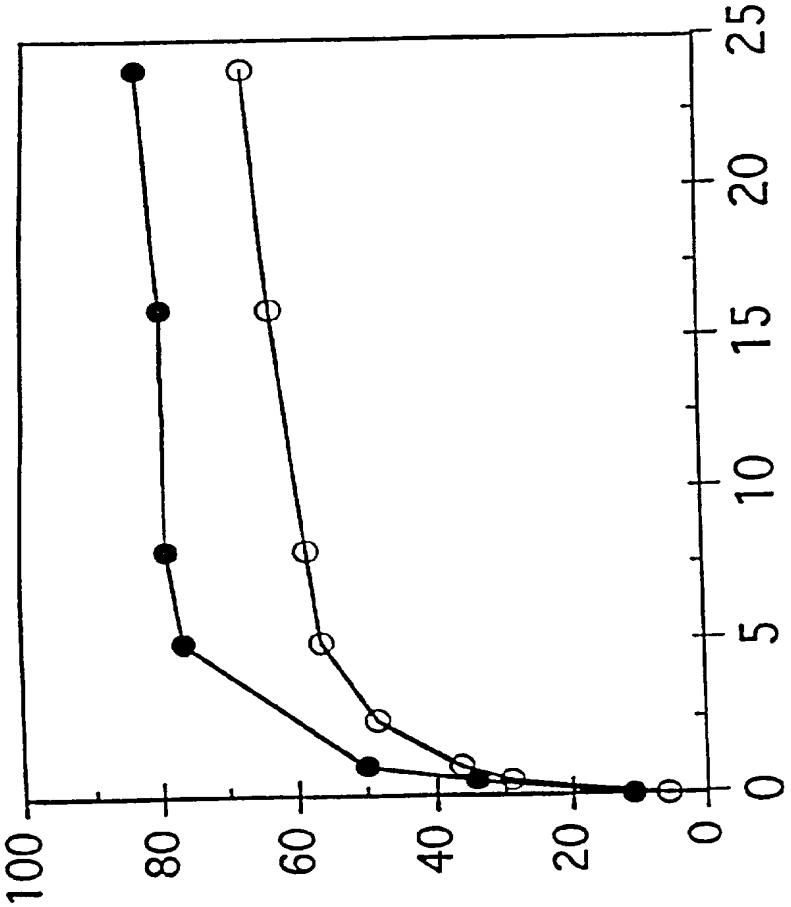

FIG. 3A

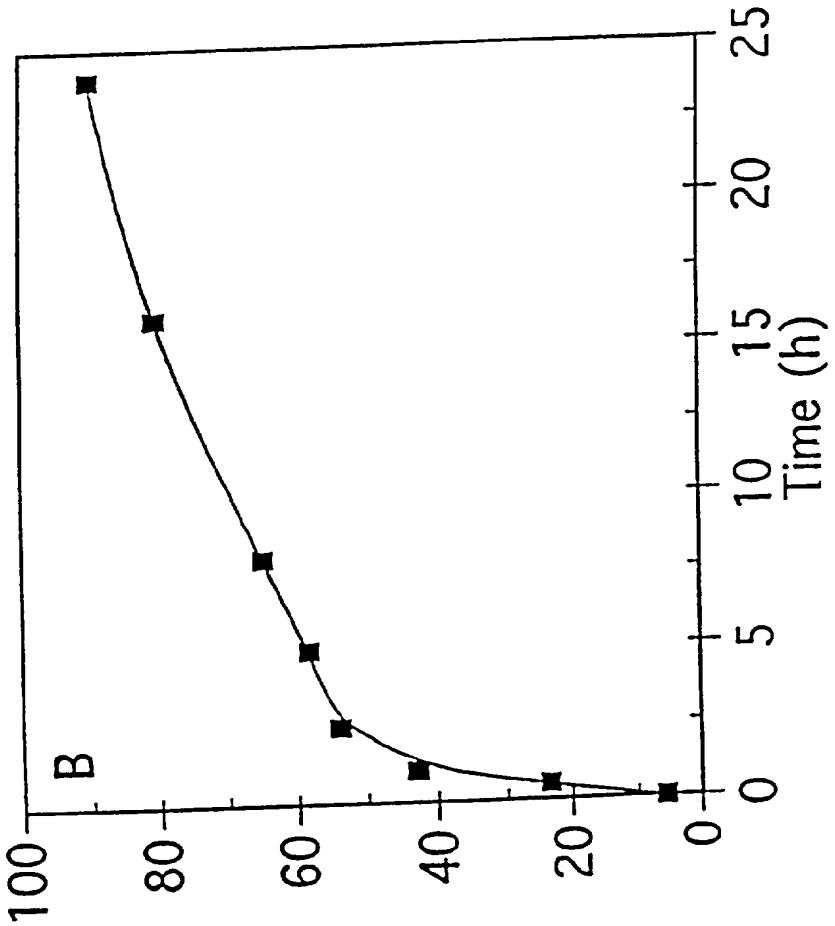

FIG. 3B

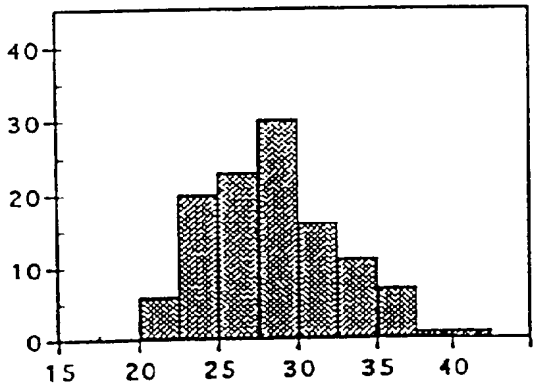

FIG. 4A

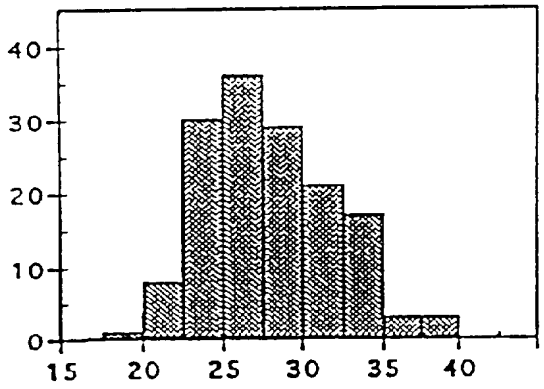

FIG. 4B

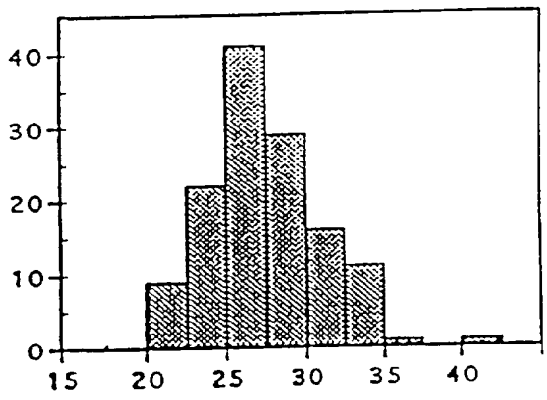

FIG. 4C

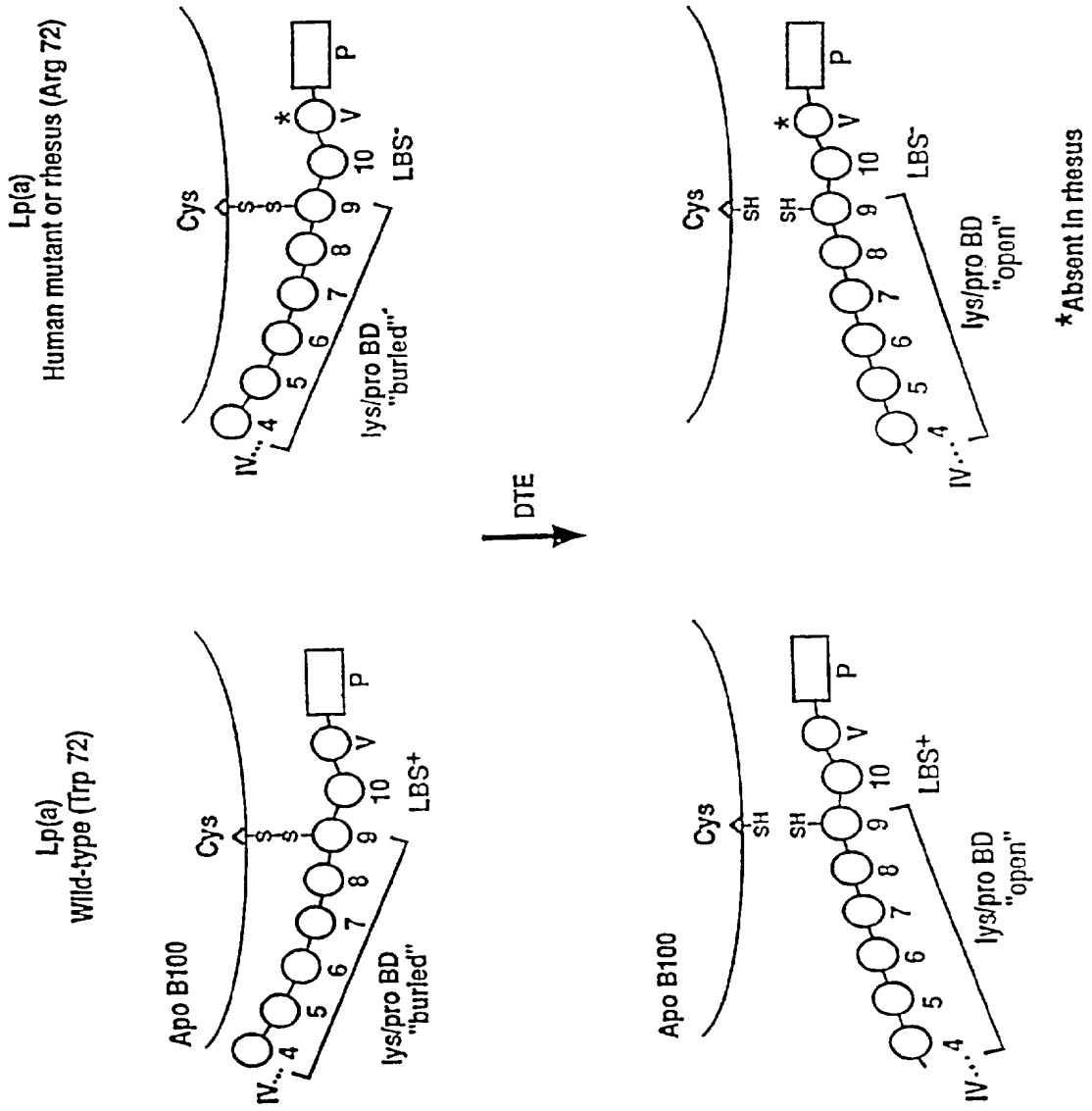
Lp(a)
Human mutant or rhesus (Arg 72)
Cys
S
S
P
V
10
9
8
7
6
5
4
IV
LBS-
lys/pro BD
"burled"
DTE
SH
*
*Absent in rhesus
Lp(a)
Wild-type (Trp 72)
Apo B100
LBS+
lys/pro BD
"open"

FIG. 5

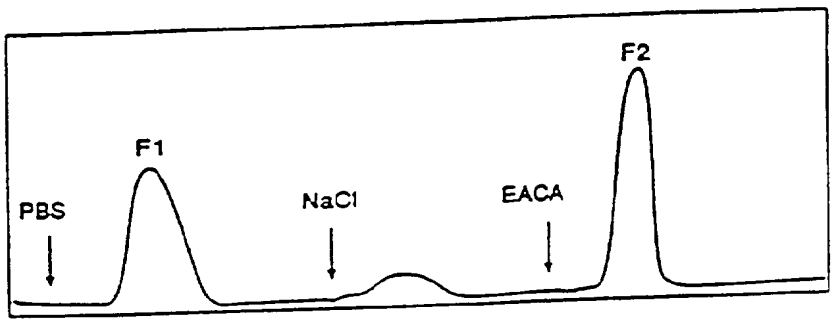

FIG. 6

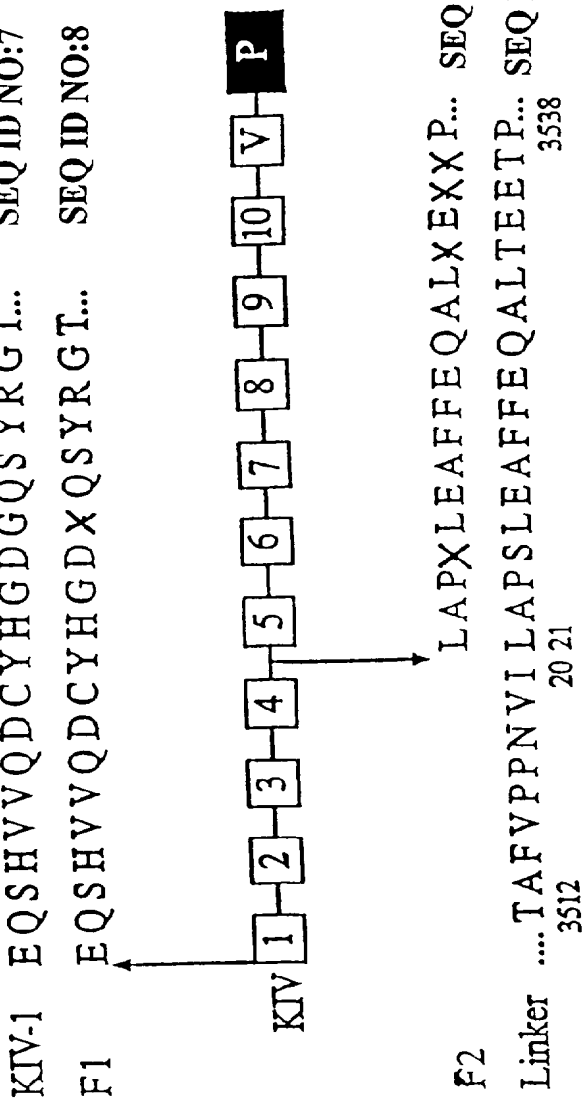
1
KIV-1 EQSHVVQDCYHGDGQSYRGT...
20
SEQ ID NO:7
F1 EQSHVVQDCYHGDXQSYRGT...
SEQ ID NO:8
KIV
1 2 3 4 5 6 7 8 9 10 V P
F2
LAPXLEAFFEQALXEXXP...
SEQ ID NO:9
Linker ....TAFVPPNVILAPSLEAFFEQALTEETP...
SEQ ID NO:10
3512
20 21
3538

FIG. 7

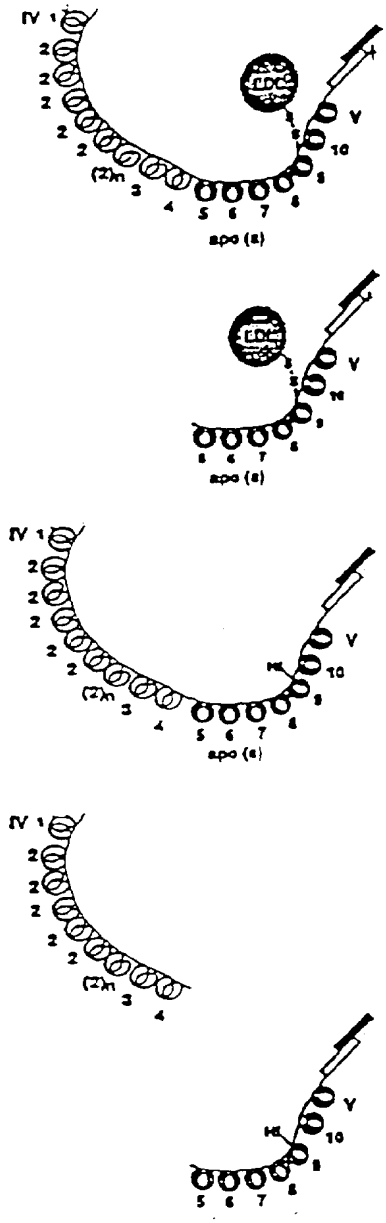

FIG. 8

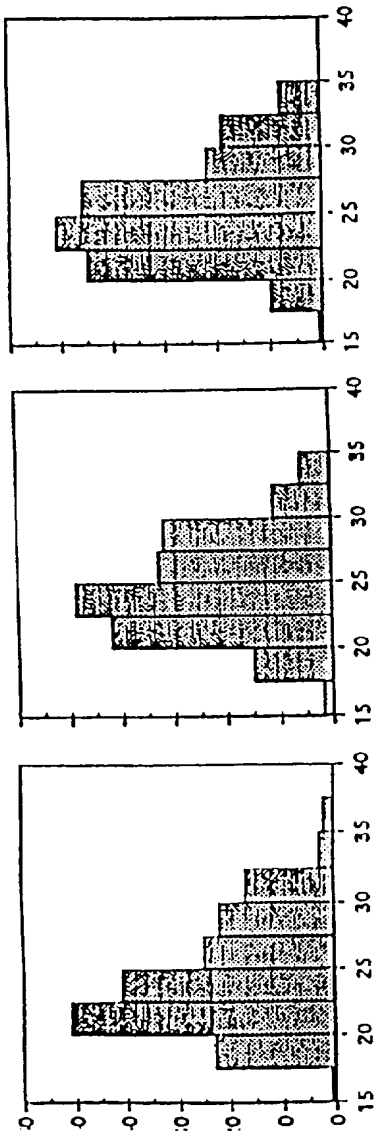

FIG. 9C

FIG. 9B

FIG. 9A

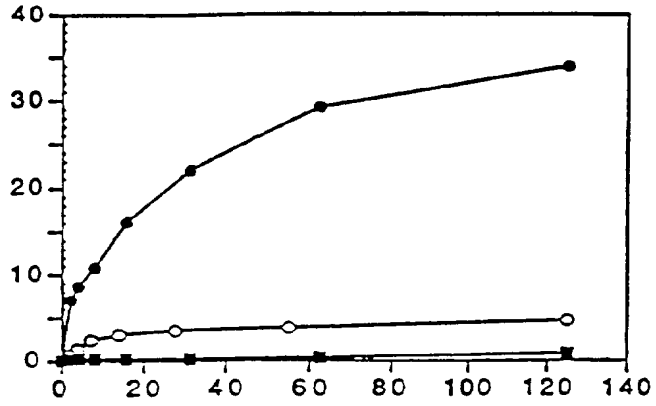

FIG. 10A

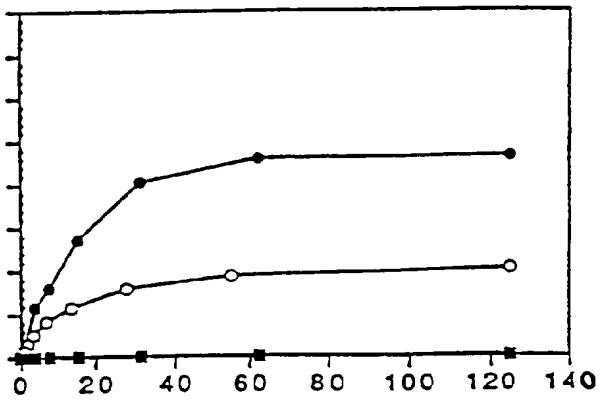

FIG. 10B

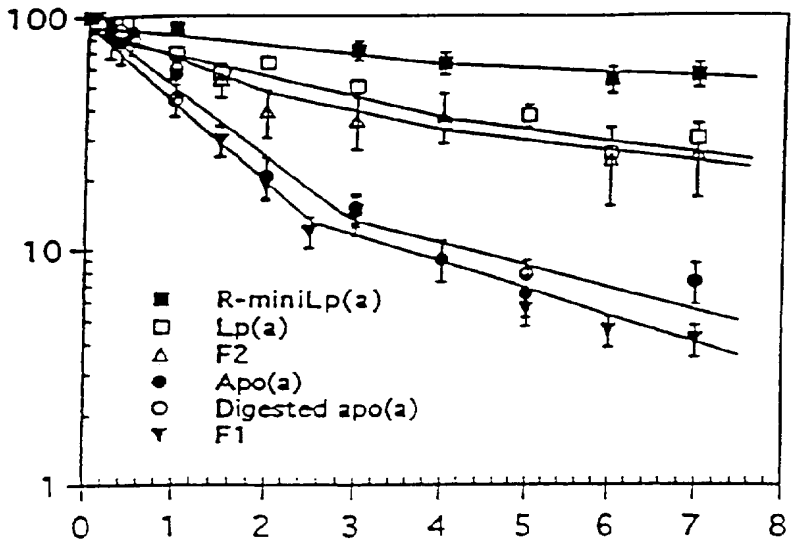

FIG. 11

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PC I/US 96/18136

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/775 A61K38/16 C07K16/18 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 659 865 A (AKZO NOBEL NV) 28 June 1995 see claim 6; example 1 ---	22,38
X	EP 0 621 284 A (SHINO-TEST CORPORATION) 26 October 1994 see page 18 - page 28 ---	22,28,38
X	WO 92 09893 A (J CARBAUGH) 11 June 1992 see the whole document --- -/--	22,28,38

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 March 1997	25.03.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCHEMISTRY, vol. 31, no. 27, 14 July 1992, EASTON, PA US, pages 6333-6339, XP002027704 D ROUY ET AL.: "Apolipoprotein(a) and plasminogen interactionss with fibrin; a study with recombinant apolipoprotein (a) and isolated plamsinogen fragments" see the whole document	1-40
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, no. 24, 15 December 1990, WASHINGTON US, pages 11643-11647, XP002027705 C BRUNNER ET AL.: "Cys4057 of apolipoprotein (a) us essential for lipoprotein assembly" cited in the application see the whole document, especially the introduction and the discussion sections	1
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 9, 25 March 1991, MD US, pages 5480-5485, XP002027706 V N TRIEU ET AL.: "Interaction of apolipoprotein (a) with apolipoprotein B-containing lipoproteins" see the whole document	1
P,X	BIOCHEMISTRY, vol. 34, no. 50, 19 December 1995, EASTON, PA US, pages 16483-16492, XP002027707 C EDELSTEIN ET AL.: "Determinants of lipoprotein (a) assembly; a study of wild-type and mutant apolipoprotein (a) phenotypes isolated from human and rhesus monkey lipoprotein (a) under mild reductive conditions" see the whole document	1-40
P,X	CHEMICAL ABSTRACTS, vol. 125, no. 17, 21 October 1996 Columbus, Ohio, US; abstract no. 217674, XP002027708 see abstract & J LIPID RESEARCH, vol. 37, no. 8, August 1996, pages 1786-1801, C EDELSTEIN ET AL.: "Functional and metabolic differences between elastase-generated fragments of human lipoprotein (a) and apolipoprotein (a)"	1-40

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ional Application No
PCT/US 96/18136

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 659865 A	28-06-95	JP 7294735 A	10-11-95
		JP 7294940 A	10-11-95
		JP 8021915 A	23-01-96
		JP 8027066 A	30-01-96
		JP 8029618 A	02-02-96
		JP 8041461 A	13-02-96
		JP 8092556 A	09-04-96
		JP 8003111 A	09-01-96
		JP 8075924 A	22-03-96
EP 621284 A	26-10-94	AU 672028 B	19-09-96
		AU 4761493 A	15-03-94
		WO 9404563 A	03-03-94
WO 9209893 A	11-06-92	AU 9088891 A	25-06-92
		US 5490981 A	13-02-96